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(54) **Title:** LIPID-BASED DRUG CARRIERS FOR RAPID PENETRATION THROUGH MUCUS LININGS

(57) **Abstract:** Mucus-penetrating liposomal nanoparticles and methods of making and using thereof are described herein. The nanoparticles contain one or more lipids, one or more PEG-conjugated lipids, and optionally one or more additional materials that physically and/or chemically stabilize the particles. The nanoparticle have an average diameter of about 100 nm to about 300 nm, preferably from about 100 nm to about 250 nm, more preferably from about 100 nm to about 200 nm. The particles are mobile in mucus. The liposomes can further contain one or more therapeutic, prophylactic, and/or diagnostic agent to be delivered to a mucosal surface, such as the CV tract, the colon, the nose, the lungs, and/or the eyes. The liposomes can further contain one or more CEST agents to allow real time imaging of the particles in a live animal. The particles may also further contain an imaging agent, such as a fluorescent label.



## **LIPID-BASED DRUG CARRIERS FOR RAPID PENETRATION THROUGH MUCUS LININGS**

### **CROSS-REFERENCE TO RELATED APPLICATIONS**

5           This application claims priority to U.S.S.N. 61/642,776 entitled  
“*Lipid-Based Drug Carriers for Rapid Penetration Through Mucus  
Linings*”, filed on May 4, 2012. The contents of this application are  
incorporated herein by reference in their entirety.

### **STATEMENT REGARDING FEDERALLY SPONSORED**

#### **10                               RESEARCH OR DEVELOPMENT**

This invention was made with Government Support under Agreement  
R01EB015031 awarded to Michael McMahon by the National Institutes of  
Health. The Government has certain rights in the invention.

### **FIELD OF THE INVENTION**

15           This invention is in the field of liposomal nanoparticles for drug  
delivery, particularly mucus-penetrating liposomal nanoparticles.

### **BACKGROUND OF THE INVENTION**

Mucus is a viscous and adhesive substance that efficiently traps and  
removes pathogens, hazard particles, and conventional drug or gene carriers  
20   from mucosal surfaces, including those that cover the female reproductive  
tract, GI tract, lung, airway and eyes. To deliver therapeutics efficiently  
across the mucosal surfaces, drug or gene carriers must be able to penetrate  
the mucus barrier to avoid the mucus-induced aggregation and rapid  
clearance.

25           Different strategies have been proposed to render drug or gene  
carriers muco-inert by covalently or non-covalently modifying particle  
surfaces with a dense coating of low molecular weight (MW) poly(ethylene  
glycol) (PEG), a hydrophilic, neutrally charged molecule that efficiently  
shields particles from strong adhesion to mucus. Prior formulations of  
30   mucus-penetrating particles (MPP) are mainly based on biodegradable  
polymers, of which the hydrophobic segment serves as the loading vehicle  
for poorly soluble drugs. However, it remains challenging to encapsulate  
water soluble agents (e.g, proteins such as antibodies or growth factors) in

hydrophobic polymeric particles, especially for those with small sizes at sub-micron scale such as MPP.

Lipid-based systems, such as liposomes, have been studied as means for effective drug delivery, particularly for hydrophilic or water-soluble agents. However, these systems are generally unable to achieve efficient transmucosal delivery mainly due to the strong hindrance by the mucus mesh. The intense interactions between delivery particles and mucus constituents might also induce unwanted decomposition of the particles or the encapsulated payloads.

There exists a need for a drug delivery systems which can rapidly penetrate mucus which overcomes the limitations discussed above associated with polymeric systems.

Therefore, there is a need to provide a drug delivery system, particularly a lipid-based system, such as liposomes, which can rapidly penetrate mucus which overcomes the limitations discussed above associated with polymeric systems and methods of making and using thereof.

### SUMMARY OF THE INVENTION

Mucus-penetrating liposomal nanoparticles and methods of making and using thereof are described herein. The nanoparticles contain one or more lipids, one or more PEG-conjugated lipids, and optionally one or more additional materials that physically and/or chemically stabilize the particles. The concentration of the PEG-conjugated lipid is from about 8 mole percent to about 30 mole percent. The liposomes can be prepared by any means known in the art. The nanoparticle have an average diameter of about 100 nm to about 300 nm, preferably from about 100 nm to about 250 nm, more preferably from about 100 nm to about 200 nm. The particles are mobile in mucus. "Mobile" as used herein means particles which travel a distance of at least over 10 times its radius (about 100 nm) in a 20 second movie, which corresponds to about  $0.1 \mu\text{m}^2/\text{sec}$ .

The liposomes can further contain one or more therapeutic, prophylactic, and/or diagnostic agent to be delivered to a mucosal surface, such as the CV tract, the colon, the nose, the lungs, and/or the eyes. The liposomes can further contain one or more CEST agents to allow real time

imaging of the particles in a live animal. The particles may also further contain an imaging agent, such as a fluorescent label. Finally, the particle may contain a mucus-disrupting agent. The particles may contain one or more of the agents listed above.

- 5           The liposomes can be combined with one or more pharmaceutically acceptable excipients to prepare pharmaceutical formulations. The liposomes can be administered by a variety of routes of administration, such as enteral or parenteral, as well as topical or pulmonary.

### BRIEF DESCRIPTION OF THE DRAWINGS

- 10           Figure 1 is a schematic of mucus-penetrating liposomal nanoparticles formed from phosphatidyl choline (PC), PEG2000-DSPE, cholesterol, and Rhodamine-PE having encapsulated therein a CEST agent (barbituric acid) and doxorubicin.

- 15           Figure 2A is a graph showing the diameter of the liposomal nanoparticles as a function of time (hours) for liposomal nanoparticles containing different amounts of polyethylene glycol (PEG). Figure 2B is a graph showing the polydispersity of the liposomal nanoparticles as a function of time (hours) for liposomal nanoparticles containing different amounts of PEG.

- 20           Figure 3 is a schematic showing the penetration of mucus-penetrating particles compared to non-mucus-penetrating particles.

Figure 4 shows the relative signal intensity as a function of frequency offset from water for liposomal nanoparticles prepared with different amounts of PEG-conjugated lipid.

### 25           DETAILED DESCRIPTION OF THE INVENTION

#### I.       Definitions

“Liposome”, as used herein, refers to vesicles or particles which possess a lipid bilayer enclosing an aqueous compartment.

- 30           “Nanoparticle,” as used herein, generally refers to a particle of any shape having an average diameter from about 1 nm up to, but not including, about 1 micron, preferably from about 5 nm to about 500 nm, most preferably from about 5 nm to about 300 nm. In some embodiments, the particles have an average diameter from about 100 nm to about 300 nm,

preferably from about 100 nm to about 250 nm, more preferably from about 100 nm to about 200 nm. Nanoparticles having a spherical shape are generally referred to as “nanospheres”.

“Mean particle size,” as used herein, generally refers to the statistical  
5 mean particle size (diameter) of the particles in a population of particles. The diameter of an essentially spherical particle may be referred to as the physical or hydrodynamic diameter. The diameter of a non-spherical particle may refer preferentially to the hydrodynamic diameter. As used herein, the diameter of a non-spherical particle may refer to the largest linear distance  
10 between two points on the surface of the particle. Mean particle size can be measured using methods known in the art, such as dynamic light scattering.

“Monodisperse” and “homogeneous size distribution,” are used interchangeably herein and describe a plurality of liposomal nanoparticles or microparticles where the particles have the same or nearly the same diameter  
15 or aerodynamic diameter. As used herein, a monodisperse distribution refers to particle distributions in which 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 86, 88, 89, 90, 91, 92, 93, 94, 95% or greater of the distribution lies within 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, or 10% of the mass median diameter or aerodynamic diameter.

20 “Mobile” as used herein means particles which travel a distance of at least over 10 times its radius (about 100 nm) in a 20 second movie, which corresponds to about  $0.1 \mu\text{m}^2/\text{sec}$ .

“Hydrophilic” as used herein refers to substances that have strongly polar groups that readily interact with water.

25 “Lipophilic” refers to compounds having an affinity for lipids.

“Amphiphilic” refers to a molecule combining hydrophilic and lipophilic (hydrophobic) properties

“Hydrophobic” as used herein refers to substances that lack an affinity for water; tending to repel and not absorb water as well as not  
30 dissolve in or mix with water.

“Pharmaceutically acceptable,” as used herein, refers to compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of

human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications commensurate with a reasonable benefit/risk ratio, in accordance with the guidelines of agencies such as the Food and Drug Administration.

5           “Biocompatible” as used herein, generally refer to materials that are, along with any metabolites or degradation products thereof, generally non-toxic to the recipient, and do not cause any significant adverse effects to the recipient. Generally speaking, biocompatible materials are materials which do not elicit a significant inflammatory or immune response when  
10 administered to a patient.

          “Molecular weight,” as used herein, generally refers to the relative average chain length of the bulk polymer, unless otherwise specified. In practice, molecular weight can be estimated or characterized using various methods including gel permeation chromatography (GPC) or capillary  
15 viscometry. GPC molecular weights are reported as the weight-average molecular weight (Mw) as opposed to the number-average molecular weight (Mn). Capillary viscometry provides estimates of molecular weight as the inherent viscosity determined from a dilute polymer solution using a particular set of concentration, temperature, and solvent conditions.

20           “Mucus,” as used herein, refers to a viscoelastic natural substance containing primarily mucin glycoproteins and other materials, which protects epithelial surface of various organs/tissues, including respiratory, nasal, cervicovaginal, gastrointestinal, rectal, visual and auditory systems.

          “Sputum,” as used herein, refers to highly viscoelastic mucus secretions  
25 consist of a variety of macromolecules such as DNA, actins and other cell debris released from dead cells in addition to mucin glycoproteins. “Sputum” is generally present in the pathogenic airways of patients afflicted by obstructive lung diseases, including but not limited to, asthma, COPD and CF. “CF mucus” and “CF sputum,” as used herein, refer to mucus and  
30 sputum, respectively, from a patient suffering from cystic fibrosis. In some embodiments, the mucus is non-ovulatory human cervicovaginal mucus (CVM).

“Mucus Degrading Agent,” as used herein, refers to a substance which increases the rate of mucus clearance when administered to a patient. Mucus degrading agents are known in the art. See, for example, Hanes, J. et al. Gene Delivery to the Lung. in Pharmaceutical Inhalation Aerosol Technology, Marcel Dekker, Inc., New York: 489-539 (2003). Examples of mucus degrading agents include N-acetylcysteine (NAC), which cleaves disulfide and sulfhydryl bonds present in mucin. Other mucus degrading agents include mugwort, bromelain, papain, clerodendrum, acetylcysteine, bromhexine, carbocysteine, eprazinone, mesna, ambroxol, sobrerol, domiodol, denufosol, letosteine, stepronin, tiopronin, gelsolin, thymosin  $\beta$ 4, nelteneine, erdosteine, and various DNases including rhDNase.

The term "surfactant" as used herein refers to an agent that lowers the surface tension of a liquid.

The term "therapeutic agent" refers to an agent that can be administered to prevent or treat a disease or disorder. Therapeutic agents can be a nucleic acid, a nucleic acid analog, a small molecule, a peptidomimetic, a protein, peptide, carbohydrate or sugar, lipid, or surfactant, or a combination thereof.

The term "treating" or preventing a disease, disorder or condition from occurring in an animal which may be predisposed to the disease, disorder and/or condition but has not yet been diagnosed as having it; inhibiting the disease, disorder or condition, e.g., impeding its progress; and relieving the disease, disorder, or condition, e.g., causing regression of the disease, disorder and/or condition. Treating the disease or condition includes ameliorating at least one symptom of the particular disease or condition, even if the underlying pathophysiology is not affected, such as treating the pain of a subject by administration of an analgesic agent even though such agent does not treat the cause of the pain.

The term "targeting moiety" as used herein refers to a moiety that localizes to or away from a specific locale. The moiety may be, for example, a protein, nucleic acid, nucleic acid analog, carbohydrate, or small molecule. The entity may be, for example, a therapeutic compound such as a small molecule, or a diagnostic entity such as a detectable label. The locale may be

a tissue, a particular cell type, or a subcellular compartment. In one embodiment, the targeting moiety directs the localization of an active entity. The active entity may be a small molecule, protein, polymer, or metal. The active entity may be useful for therapeutic, prophylactic, or diagnostic purposes.

5 The term "therapeutically effective amount" refers to an amount of the therapeutic agent that, when incorporated into and/or onto particles described herein, produces some desired effect at a reasonable benefit/risk ratio applicable to any medical treatment. The effective amount may vary depending on such factors as the disease or condition being treated, the particular targeted constructs being administered, the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art may empirically determine the effective amount of a particular compound without necessitating undue experimentation.

15 The terms "incorporated" and "encapsulated" refers to incorporating, formulating, or otherwise including an active agent into and/or onto a composition that allows for release, such as sustained release, of such agent in the desired application. The terms contemplate any manner by which a therapeutic agent or other material is incorporated into a polymer matrix, including, for example: attached to a monomer of such polymer (by covalent, ionic, or other binding interaction), physical admixture, enveloping the agent in a coating layer of polymer, incorporated into the polymer, distributed throughout the polymeric matrix, appended to the surface of the polymeric matrix (by covalent or other binding interactions), encapsulated inside the polymeric matrix, etc. The term "co-incorporation" or "co-encapsulation" refers to the incorporation of a therapeutic agent or other material and at least one other therapeutic agent or other material in a subject composition.

## II. Mucus-penetrating liposomal particles

Mucus-penetrating liposomal particles are described herein. A liposome is a synthetic vesicle or particle containing a lipid bilayer. Liposomes can be used for drug delivery. The major types of liposomes are the multilamellar vesicle (MLV), the small unilamellar vesicle (SUV), the large unilamellar vesicle (LUV), and the cochleate vesicle.



Liposomes have been pegylated, e.g., prepared from PEG-conjugated lipids, to prepare stealth liposomes for intravenous administration. The PEG extends the blood circulation time which reduces mononuclear phagocyte system uptake, i.e., stealth liposomes. Such liposomes typically contain  
5 about 3-7 mole percent PEG-conjugated lipids. In contrast, the liposomes described herein are mucus penetrating. It has been found that significantly higher concentrations of PEG-conjugated lipids are necessary to impart mucus-penetrating properties. The concentration of PEG-conjugated lipids is typically at least about 8 or about 10 mole percent, for example from about  
10 8 or about 10 to about 30 mole percent, preferably from about 8 or about 10 mole percent to about 20 mole percent.

#### **A. Lipids**

The liposomes described herein contain one or more lipid components. Lipids are naturally occurring, synthetic, or semi-synthetic  
15 molecules that include fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, triglycerides, phospholipids, and others.

Lipids may be broadly defined as hydrophobic or amphiphilic small molecules; the amphiphilic nature of some lipids allows them to form  
20 structures such as vesicles, liposomes, or membranes in an aqueous environment. Biological lipids originate entirely or in part from two distinct types of biochemical subunits or "building-blocks": ketoacyl and isoprene groups.

Using this approach, lipids may be divided into eight categories: fatty  
25 acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides (derived from condensation of ketoacyl subunits); and sterol lipids and prenol lipids (derived from condensation of isoprene subunits).

The concentration of the lipid is from about 25 to 99 mole percent, preferably about 25 to about 95 mole percent, more preferably from about 20  
30 to about 90 mole percent, most preferably from about 20 to about 80 mole percent. In some embodiments, the concentration may be from about 40 to about 70 mole percent, preferably from about 40 to about 60 mole percent.

### 1. Fatty acids

Fatty acids, or fatty acid residues when they form part of a lipid, are a diverse group of molecules which can be prepared synthetically or synthesized naturally by chain-elongation of an acetyl-CoA primer with malonyl-CoA or methylmalonyl-CoA groups in a process called fatty acid synthesis. Fatty acids are made of a hydrocarbon chain that terminates with a carboxylic acid group; this arrangement confers the molecule with a polar, hydrophilic end, and a nonpolar, hydrophobic end that is insoluble in water. The carbon chain, typically between four and 24 carbons long, may be saturated or unsaturated, and may be attached to functional groups containing oxygen, halogens, nitrogen, and sulfur. Where a double bond exists, there is the possibility of either a cis or trans geometric isomerism, which significantly affects the molecule's configuration. Cis-double bonds cause the fatty acid chain to bend, an effect that is compounded with more double bonds in the chain. Most naturally occurring fatty acids are of the cis configuration, although the trans form does exist in some natural and partially hydrogenated fats and oils. Other major lipid classes in the fatty acid category are the fatty esters and fatty amides.

### 2. Glycerolipids

Glycerolipids are composed mainly of mono-, di-, and tri-substituted glycerols, the most well-known being the fatty acid triesters of glycerol, called triglycerides. The word "triacylglycerol" is sometimes used synonymously with "triglyceride", though the latter lipids contain no hydroxyl group. In these compounds, the three hydroxyl groups of glycerol are each esterified, typically by different fatty acids.

Additional subclasses of glycerolipids are represented by glycosylglycerols, which are characterized by the presence of one or more sugar residues attached to glycerol via a glycosidic linkage. Examples of structures in this category are the digalactosyldiacylglycerols found in plant membranes and seminolipid from mammalian sperm cells.

### 3. Glycerophospholipids

Glycerophospholipids, usually referred to as phospholipids, are

ubiquitous in nature and are key components of the lipid bilayer of cells, as well as being involved in metabolism and cell signaling. Most phospholipids contain a diglyceride, a phosphate group, and a simple organic molecule such as choline; one exception to this rule is sphingomyelin, which is derived  
5 from sphingosine instead of glycerol.

The structure of the phospholipid molecule generally consists of hydrophobic tails and a hydrophilic head. The 'head' is attracted to water, while the hydrophobic 'tails' are repelled by water and are forced to aggregate. The hydrophilic head contains the negatively charged phosphate  
10 group, and may contain other polar groups. The hydrophobic tail usually consists of long fatty acid hydrocarbon chains. When placed in water, phospholipids form a variety of structures depending on the specific properties of the phospholipid. Lipid bilayers occur when hydrophobic tails line up against one another, forming a membrane of hydrophilic heads on  
15 both sides facing the water.

Glycerophospholipids may be subdivided into distinct classes, based on the nature of the polar headgroup at the sn-3 position of the glycerol backbone in eukaryotes and eubacteria, or the sn-1 position in the case of  
archaeobacteria. Examples of glycerophospholipids found in biological  
20 membranes are phosphatidylcholine (also known as PC, GPCCho or lecithin), phosphatidylethanolamine (PE or GPEtn) and phosphatidylserine (PS or GPSer).

In eukaryotes, phospholipids are generally classified into two types: diacylglycerides and phosphingolipids. Examples of diacylglycerides  
25 include, but are not limited to, phosphatidic acid (phosphatidate) (PA), phosphatidylethanolamine (cephalin) (PE), phosphatidylcholine (lecithin) (PC), phosphatidylserine (PS), and phosphoinositides, such as phosphatidylinositol (PI), phosphatidylinositol phosphate (PIP), phosphatidylinositol biphosphate (PIP2) and, phosphatidylinositol  
30 triphosphate (PIP3). Examples of phosphingolipids include, but are not limited to, ceramide phosphorylcholine (Sphingomyelin) (SPH), ceramide phosphorylethanolamine (Sphingomyelin) (Cer-PE), and Ceramide phosphoryllipid.

Other phospholipids that can be used are shown in Table 1 below.

Table 1. Phospholipids			
<u>Abbreviation</u>	<u>CAS No.</u>	<u>Name</u>	<u>Type</u>
DDPC	3436-44-0	1,2-Didecanoyl- <i>sn</i> -glycero-3-phosphocholine	Phosphatidylcholine
DEPA-NA	80724-31-8	1,2-Dierucoyl- <i>sn</i> -glycero-3-phosphate (Sodium Salt)	Phosphatidic acid
DEPC	56649-39-9	1,2-Dierucoyl- <i>sn</i> -glycero-3-phosphocholine	Phosphatidylcholine
DEPE	988-07-2	1,2-Dierucoyl- <i>sn</i> -glycero-3-phosphoethanolamine	Phosphatidylethanolamine
DEPG-NA		1,2-Dierucoyl- <i>sn</i> -glycero-3[Phospho-rac-(1-glycerol...)] (Sodium Salt)	Phosphatidylglycerol
DLOPC	998-06-1	1,2-Dilinoleoyl- <i>sn</i> -glycero-3-phosphocholine	Phosphatidylcholine
DLPA-NA		1,2-Dilauroyl- <i>sn</i> -glycero-3-phosphate (Sodium Salt)	Phosphatidic acid
DLPC	18194-25-7	1,2-Dilauroyl- <i>sn</i> -glycero-3-phosphocholine	Phosphatidylcholine
DLPE		1,2-Dilauroyl- <i>sn</i> -glycero-3-phosphoethanolamine	Phosphatidylethanolamine
DLPG-NA		1,2-Dilauroyl- <i>sn</i> -glycero-3[Phospho-rac-(1-glycerol...)] (Sodium Salt)	Phosphatidylglycerol
DLPG-NH4		1,2-Dilauroyl- <i>sn</i> -glycero-3[Phospho-rac-(1-glycerol...)] (Ammonium Salt)	Phosphatidylglycerol
DLPS-NA		1,2-Dilauroyl- <i>sn</i> -glycero-3-phosphoserine (Sodium Salt)	Phosphatidylserine

DMPA-NA	80724-3	1,2-Dimyristoyl- <i>sn</i> -glycero-3-phosphate (Sodium Salt)	Phosphatidic acid
DMPC	18194-24-6	1,2-Dimyristoyl- <i>sn</i> -glycero-3-phosphocholine	Phosphatidylcholine
DMPE	988-07-2	1,2-Dimyristoyl- <i>sn</i> -glycero-3-phosphoethanolamine	Phosphatidylethanolamine
DMPG-NA	67232-80-8	1,2-Dimyristoyl- <i>sn</i> -glycero-3[Phospho-rac-(1-glycerol...)] (Sodium Salt)	Phosphatidylglycerol
DMPG-NH4		1,2-Dimyristoyl- <i>sn</i> -glycero-3[Phospho-rac-(1-glycerol...)] (Ammonium Salt)	Phosphatidylglycerol
DMPG-NH4/NA		1,2-Dimyristoyl- <i>sn</i> -glycero-3[Phospho-rac-(1-glycerol...)] (Sodium/Ammonium Salt)	Phosphatidylglycerol
DMPS-NA		1,2-Dimyristoyl- <i>sn</i> -glycero-3-phosphoserine (Sodium Salt)	Phosphatidylserine
DOPA-NA		1,2-Dioleoyl- <i>sn</i> -glycero-3-phosphate (Sodium Salt)	Phosphatidic acid
DOPC	4235-95-4	1,2-Dioleoyl- <i>sn</i> -glycero-3-phosphocholine	Phosphatidylcholine
DOPE	4004-5-1-	1,2-Dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine	Phosphatidylethanolamine
DOPG-NA	62700-69-0	1,2-Dioleoyl- <i>sn</i> -glycero-3[Phospho-rac-(1-glycerol...)] (Sodium Salt)	Phosphatidylglycerol
DOPS-NA	70614-14-1	1,2-Dioleoyl- <i>sn</i> -glycero-3-phosphoserine (Sodium Salt)	Phosphatidylserine

DPPA-NA	71065-87-7	1,2-Dipalmitoyl- <i>sn</i> -glycero-3-phosphate (Sodium Salt)	Phosphatidic acid
DPPC	63-89-8	1,2-Dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine	Phosphatidylcholine
DPPE	923-61-5	1,2-Dipalmitoyl- <i>sn</i> -glycero-3-phosphoethanolamine	Phosphatidylethanolamine
DPPG-NA	67232-81-9	1,2-Dipalmitoyl- <i>sn</i> -glycero-3[Phospho-rac-(1-glycerol...)] (Sodium Salt)	Phosphatidylglycerol
DPPG-NH4	73548-70-6	1,2-Dipalmitoyl- <i>sn</i> -glycero-3[Phospho-rac-(1-glycerol...)] (Ammonium Salt)	Phosphatidylglycerol
DPPS-NA		1,2-Dipalmitoyl- <i>sn</i> -glycero-3-phosphoserine (Sodium Salt)	Phosphatidylserine
DSPA-NA	108321-18-2	1,2-Distearoyl- <i>sn</i> -glycero-3-phosphate (Sodium Salt)	Phosphatidic acid
DSPC	816-94-4	1,2-Distearoyl- <i>sn</i> -glycero-3-phosphocholine	Phosphatidylcholine
DSPE	1069-79-0	1,2-Distearoyl- <i>sn</i> -glycero-3-phosphoethanolamine	Phosphatidylethanolamine
DSPG-NA	67232-82-0	1,2-Distearoyl- <i>sn</i> -glycero-3[Phospho-rac-(1-glycerol...)] (Sodium Salt)	Phosphatidylglycerol
DSPG-NH4	108347-80-4	1,2-Distearoyl- <i>sn</i> -glycero-3[Phospho-rac-(1-glycerol...)] (Ammonium Salt)	Phosphatidylglycerol
DSPS-NA		1,2-Distearoyl- <i>sn</i> -glycero-3-phosphoserine (Sodium Salt)	Phosphatidylserine

Egg  
 Sphingomyelin  
 empty  
 Liposome

EPC		Egg-PC	Phosphatidylcholine
HEPC		Hydrogenated Egg PC	Phosphatidylcholine
HSPC		High purity Hydrogenated Soy PC	Phosphatidylcholine
HSPC		Hydrogenated Soy PC	Phosphatidylcholine
LYSOPC	18194-	1-Myristoyl- <i>sn</i> -glycero-	Lysophosphatidylcholine
MYRISTIC	24-6	3-phosphocholine	
LYSOPC	17364-	1-Palmitoyl- <i>sn</i> -glycero-	Lysophosphatidylcholine
PALMITIC	16-8	3-phosphocholine	
LYSOPC	19420-	1-Stearoyl- <i>sn</i> -glycero-	Lysophosphatidylcholine
STEARIC	57-6	3-phosphocholine	
Milk Sphingomyelin MPPC		1-Myristoyl-2- palmitoyl- <i>sn</i> -glycero 3- phosphocholine	Phosphatidylcholine
MSPC		1-Myristoyl-2-stearoyl- <i>sn</i> -glycero-3- phosphocholine	Phosphatidylcholine
PMPC		1-Palmitoyl-2- myristoyl- <i>sn</i> -glycero-3- phosphocholine	Phosphatidylcholine
POPC	26853- 31-6	1-Palmitoyl-2-oleoyl- <i>sn</i> -glycero-3- phosphocholine	Phosphatidylcholine
POPE		1-Palmitoyl-2-oleoyl- <i>sn</i> -glycero-3- phosphoethanolamine	Phosphatidylethanolamine
POPG-NA	81490- 05-3	1-Palmitoyl-2-oleoyl- <i>sn</i> -glycero-3[Phospho- rac-(1-glycerol)...] (Sodium Salt)	Phosphatidylglycerol
PSPC		1-Palmitoyl-2-stearoyl- <i>sn</i> -glycero-3- phosphocholine	Phosphatidylcholine
SMPC		1-Stearoyl-2-myristoyl- <i>sn</i> -glycero-3- phosphocholine	Phosphatidylcholine
SOPC		1-Stearoyl-2-oleoyl- <i>sn</i> - glycero-3- phosphocholine	Phosphatidylcholine
SPPC		1-Stearoyl-2-palmitoyl- <i>sn</i> -glycero-3- phosphocholine	Phosphatidylcholin

In some embodiments, the lipid component is one or more phospholipids. In some embodiments, the lipid is, or contains, phosphatidylcholine (PC, e.g., egg PC or hydrogenated soy PC). The concentration of the phospholipid is from about 25 to 99 mole percent, preferably about 25 to about 95 mole percent, more preferably from about 20 to about 90 mole percent, most preferably from about 20 to about 80 mole percent. In some embodiments, the concentration may be from about 40 to about 70 mole percent, preferably from about 40 to about 60 mole percent.

#### 4. Sphingolipids

Sphingolipids are a complicated family of compounds that share a common structural feature, a sphingoid base backbone that is synthesized de novo from the amino acid serine and a long-chain fatty acyl CoA, then converted into ceramides, phosphosphingolipids, glycosphingolipids and other compounds. The major sphingoid base of mammals is commonly referred to as sphingosine. Ceramides (N-acyl-sphingoid bases) are a major subclass of sphingoid base derivatives with an amide-linked fatty acid. The fatty acids are typically saturated or mono-unsaturated with chain lengths from 16 to 26 carbon atoms.

The major phosphosphingolipids of mammals are sphingomyelins (ceramide phosphocholines), whereas insects contain mainly ceramide phosphoethanolamines and fungi have phytoceramide phosphoinositols and mannose-containing headgroups. The glycosphingolipids are a diverse family of molecules composed of one or more sugar residues linked via a glycosidic bond to the sphingoid base. Examples of these are the simple and complex glycosphingolipids such as cerebroside and gangliosides.

#### 5. Sterol lipids

Sterol lipids, such as cholesterol and its derivatives, are an important component of membrane lipids, along with the glycerophospholipids and sphingomyelins. The steroids are all derived from the same fused four-ring core structure. Other examples of sterols are the bile acids and their conjugates. The plant equivalents are the phytosterols, such as  $\beta$ -sitosterol, stigmasterol, and brassicasterol.



## 6. Prenol lipids

Prenol lipids are synthesized from the five-carbon-unit precursors isopentenyl diphosphate and dimethylallyl diphosphate. The simple isoprenoids (linear alcohols, diphosphates, etc.) are formed by the successive  
5 addition of C5 units, and are classified according to number of these terpene units. Structures containing greater than 40 carbons are known as polyterpenes. Carotenoids are important simple isoprenoids that function as antioxidants and as precursors of vitamin A. Another biologically important class of molecules is exemplified by the quinones and hydroquinones, which  
10 contain an isoprenoid tail attached to a quinonoid core of non-isoprenoid origin. Vitamin E and vitamin K, as well as the ubiquinones, are examples of this class. Prokaryotes synthesize polyprenols (called bactoprenols) in which the terminal isoprenoid unit attached to oxygen remains unsaturated, whereas in animal polyprenols (dolichols) the terminal isoprenoid is reduced.

## 7. Saccharolipids

Saccharolipids describe compounds in which fatty acids are linked directly to a sugar backbone, forming structures that are compatible with membrane bilayers. In the saccharolipids, a monosaccharide substitutes for the glycerol backbone present in glycerolipids and glycerophospholipids.  
20 The most familiar saccharolipids are the acylated glucosamine precursors of the Lipid A component of the lipopolysaccharides in Gram-negative bacteria. Typical lipid A molecules are disaccharides of glucosamine, which are derivatized with as many as seven fatty-acyl chains.

### B. PEG-conjugated lipids

25 The liposomes also contain a PEG-conjugated lipid. The lipid can be conjugated to PEG itself or a copolymer containing PEG, such as PEO-PPO copolymer available under the tradename PLURONIC. Other materials, such as polymers, surfactants, nucleic acids, proteins, etc., can be used in place of or in combination with PEG provided the material enhances or  
30 facilitate diffusion through mucus.

The PEG-conjugated lipid can be a pegylated form of any of the lipids discussed above. In some embodiments, the PEG-conjugated lipid is a PEG-conjugated phospholipid. In particular embodiments, the PEG-

conjugated phospholipid is PEG-phosphatidylethanolamine (PEG-PE). The concentration of the PEG-conjugated lipid in the liposome is at least about 10 mole percent, for example, about 10 to about 30 mole percent, preferably about 10 mole percent to about 20 mole percent.

5           The molecular weight of PEG can vary. In some embodiments, the molecular weight of PEG is from about 500 Daltons to about 10,000 Daltons, preferably from about 500 Daltons to about 5,000 Daltons, more preferably from about 1,000 Daltons to about 5,000 Daltons, most preferably from about 2,000 Daltons to about 4,000 Daltons. In particular embodiments, the  
10          molecular weight of PEG is about 2000 Daltons.

### **C.     Stabilizer**

          The liposome can also contain one or more stabilizers. Stabilizers are components or additional components in the liposomes that reduce or prevent vesicle destabilization and/or opsonization and concomitant release  
15       of encapsulated agents or drugs. For example, stabilizers, such as cholesterol and other materials, enhance the mechanical strength of the lipid bilayer. Other materials include one or more of the lipids described above.

          The concentration of the stabilizer(s) is at least about 5 mole percent, preferably at least about 10 mole percent, more preferably at least about 20  
20       mole percent, most preferably at least about 30 mole percent. In some embodiments, the concentration of the stabilizer is from about 5 mole % to about 50 mole %. In particular embodiment, the concentration of the stabilizer is about 25, 50, or 70 mole percent. In a more particular embodiment, the concentration of the stabilizer is about 25 mole percent.

25       In some embodiments, the stabilizer is cholesterol and is present in a concentration as described above. Other suitable stabilizers include ganglioside G<sub>M1</sub>. In other embodiments, the stabilizer can be the PEG-conjugated lipid and thus an additional stabilizer or stabilizers is not required.

### **30       D.     Properties of liposomal nanoparticles**

#### **1.     Surface density of polyethylene glycol (PEG)**

          Surface density of poly(ethylene glycol) (PEG) on nanoparticles is a key parameter in determining their successful applications *in-vivo*. The

controlled delivery of drugs to mucosal surfaces is challenging because of the presence of the protective mucus layer, and the mucus-penetrating liposomal particles show promise at improved drug distribution, retention and efficacy at mucosal surfaces. The dense coating of PEG on  
5 biodegradable liposomal nanoparticles can allow rapid penetration through mucus because of the greatly reduced adhesive interaction between mucus constituents and liposomal nanoparticles.

The concentration of PEG-conjugated lipid is discussed above with reference to the mole percent of PEG-conjugated lipid. The amount of PEG  
10 can also be described in terms of surface density. Nuclear magnetic resonance (NMR) can be used to assess the surface PEG density on PEG-containing liposomal nanoparticles described herein, both qualitatively and quantitatively (PEG peak typically observed ~3.65 ppm). In some embodiments, PEG surface density can be controlled by preparing the  
15 particles from a mixture of pegylated and non-pegylated lipids. For example, the surface density of PEG on liposomal nanoparticles can be precisely controlled by preparing particles from a mixture of PEG-conjugated lipid and non-PEG-conjugated lipid. Quantitative  $^1\text{H}$  nuclear magnetic resonance (NMR) can be used to measure the surface PEG density  
20 on liposomal nanoparticles. Multiple particle tracking in human mucus and the study of mucin binding and tissue distribution in mouse vagina revealed that there exists a PEG density threshold, which is approximately 10-20 mole percent, for the liposomal nanoparticles to be effective in penetrating mucus. This density threshold may vary depending on a variety of factors including  
25 the lipid or lipids used to prepare the particles, particle size, and/or molecular weight of PEG.

The density of the coating can be varied based on a variety of factors including the surface altering material and the composition of the particle. In one embodiment, the density of the surface altering material, such as PEG, as  
30 measured by  $^1\text{H}$  NMR is at least, 0.1, 0.2, 0.5, 0.8, 1, 2, 5, 8, 10, 15, 20, 25, 40, 50, 60, 75, 80, 90, or 100 chains per  $\text{nm}^2$ . The range above is inclusive of all values from 0.1 to 100 units per  $\text{nm}^2$ . In particular embodiments, the density of the surface altering material, such as PEG, is from about 1 to

about 25 chains/nm<sup>2</sup>, from about 1 to about 20 chains/nm<sup>2</sup>, from about 5 to about 20 chains/nm<sup>2</sup>, from about 5 to about 18 chains/nm<sup>2</sup>, from about 5 to about 15 chains/nm<sup>2</sup>, or from about 10 to about 15 chains/nm<sup>2</sup>. The concentration of the surface altering material, such as PEG, can also be varied. In particular embodiments, the density of the surface-altering material (e.g., PEG) is such that the surface-altering material (e.g. PEG) adopted an extended brush configuration. In other embodiments, the mass of the surface-altering moiety is at least 1/10,000, 1/7500, 1/5000, 1/4000, 1/3400, 1/2500, 1/2000, 1/1500, 1/1000, 1/750, 1/500, 1/250, 1/200, 1/150, 1/100, 1/75, 1/50, 1/25, 1/20, 1/5, 1/2, or 9/10 of the mass of the particle. The range above is inclusive of all vales from 1/10,000 to 9/10.

## 2. Ability to penetrate mucus

The liposomal nanoparticles described herein exhibit enhanced transport through mucus, such as non-ovulatory human cervicovaginal mucus (CVM). In some embodiments, the liposomal nanoparticles travel through mucus, such as human cervicovaginal mucus, at certain absolute diffusivities. In certain embodiments, the particles may travel at diffusivities of at least  $1 \times 10^4$ ,  $2 \times 10^4$ ,  $5 \times 10^4$ ,  $1 \times 10^3$ ,  $2 \times 10^3$ ,  $5 \times 10^3$ ,  $1 \times 10^2$ ,  $2 \times 10^2$ ,  $4 \times 10^2$ ,  $5 \times 10^2$ ,  $6 \times 10^2$ ,  $8 \times 10^2$ ,  $1 \times 10^1$ ,  $2 \times 10^1$ ,  $5 \times 10^1$ , 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, or  $0.1 \mu\text{m}^2/\text{s}$  at a time scale of 1 s. In contrast, non-penetrating particles have a diffusivity of at least about  $1 \times 10^{-4} \mu\text{m}^2/\text{s}$ .

The ability of the particles to diffuse through mucus can also be evaluated qualitatively by visual inspection. In some embodiments, the concentration of PEG is about 10 mole percent and at least 50, 60, 70, 80, or 90% of the particles are mobile in non-ovulatory CVM at 2 hours and at least 30, 40, 50, 60, or 70% of the particles are mobile in non-ovulatory CVM at 15 hours. The particles exhibit little or no aggregation. In other embodiments, the concentration of PEG-conjugated lipid is about 20% and at least about 75, 80, 85, 90, 95, 96, 96, 98, or 99% of the particles are mobile in non-ovulatory CVM at 2 hours and at least about 80, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the particles are mobile in non-ovulatory CVM at 15 hours. The particles are well dispersed with little or no aggregation.

In certain embodiments, the particles comprising a surface-altering agent that inhibits the adsorption of fluorescently labeled avidin, wherein the particle adsorbs less than 99%, 95%, 90%, 70%, 50%, 40%, 30%, 20%, 15%, 10%, 8%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of  
5 the amount of fluorescently labeled avidin that is adsorbed by a corresponding particle lacking the surface-altering agent, as calculated by average maximum fluorescent intensity.

In certain embodiments, the particles contain a surface-altering agent that affects the zeta-potential of the particle, wherein the zeta potential of  
10 said particle is between -100 mv and 10 mv, between -50 mv and 10 mv, between -25 mv and 10 mv, between -20 mv and 5 mv, between -10 mv and 10 mv, between -10 mv and 5 mv, between -5 mv and 5 mv, or even between -2 mv and 2 mv.

### 3. Stability

15 The mucus-penetrating liposomal nanoparticles are physically and chemically stable. "Physically stable", as used herein, means that the particle size and/or polydispersity remain constant over an extended period of time.

In some embodiments, "physically stable" means the change in the  
20 average diameter of the particle is less than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1% over, 2, 4, 6, 8, 12, 16, 20, 24, 30, 36, or 48 hours. In particular embodiments, the change in the average diameter of the particles is less than 10, 9, 8, 7, 6, 5, 4, 3, or 2% after 48 hours.

In other embodiments, "physically stable" means the change in the  
25 polydispersity of the particle is less than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1% over, 2, 4, 6, 8, 12, 16, 20, 24, 30, 36, or 48 hours. In particular embodiments, the change in the polydispersity of the particles is less than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1% after 48 hours.

The particles preferably show little or no aggregation, remaining well  
30 dispersed when introduced into mucus.

**E. Therapeutic, prophylactic, and/or diagnostic agents****1. Therapeutic agents**

In some embodiments, the particles have encapsulated therein, dispersed therein, and/or covalently or non-covalently associate with the surface one or more therapeutic agents. The therapeutic agent can be a small molecule, protein, polysaccharide or saccharide, nucleic acid molecule and/or lipid. In some embodiments, the agent or agents are hydrophilic or water-soluble.

**i. Small molecule therapeutic agents**

Exemplary classes of small molecule therapeutic agents include, but are not limited to, analgesics, anti-inflammatory drugs, antipyretics, antidepressants, antiepileptics, antipsychotic agents, neuroprotective agents, anti-proliferatives, such as anti-cancer agent, anti-infectious agents, such as antibacterial agents and antifungal agents, antihistamines, antimigraine drugs, antimuscarinics, anxiolytics, sedatives, hypnotics, antipsychotics, bronchodilators, anti-asthma drugs, cardiovascular drugs, corticosteroids, dopaminergics, electrolytes, gastro-intestinal drugs, muscle relaxants, nutritional agents, vitamins, parasympathomimetics, stimulants, anorectics and anti-narcoleptics. Nutraceuticals can also be incorporated. These may be vitamins, supplements such as calcium or biotin, or natural ingredients such as plant extracts or phytohormones.

In one embodiment, the liposomes contain an anti-tumor agent. Classes of antitumor agents include, but are not limited to, angiogenesis inhibitors, DNA intercalators/crosslinkers, DNA synthesis inhibitors, DNA-RNA transcription regulators, enzyme inhibitors, gene regulators, microtubule inhibitors, and other antitumor agents.

Examples of angiogenesis inhibitors include, but are not limited to, Angiostatin K1-3, DL- $\alpha$ -Difluoromethyl-ornithine, Endostatin, Fumagillin, Genistein, Minocycline, Staurosporine, ( $\pm$ )-Thalidomide, revlimid, and analogs and derivatives thereof.

Examples of DNA intercalators/cross-linkers include, but are not limited to, Bleomycin, Carboplatin, Carmustine, Chlorambucil,

Cyclophosphamide, cis-Diammineplatinum(II) dichloride (Cisplatin),  
Melphalan, Mitoxantrone, Oxaliplatin, analogs and derivatives thereof.

Examples of DNA-RNA transcription regulators include, but are not  
limited to, Actinomycin D, Daunorubicin, Doxorubicin, Homoharringtonine,  
5 Idarubicin, and analogs and derivatives thereof.

Examples of enzyme inhibitors include, but are not limited to, S(+)-  
Camptothecin, Curcumin, (-)-Deguelin, 5,6-Dichlorobenz-imidazole 1- $\beta$ -D-  
ribofuranoside, Etoposide, Formestane, Fostriecin, Hispidin, 2-Imino-1-  
imidazoli-dineacetic acid (Cyclocreatine), Mevinolin, Trichostatin A,  
10 Tyrphostin AG 34, Tyrphostin AG 879, and analogs and derivatives thereof.

Examples of gene regulators include, but are not limited to, 5-Aza-2'-  
deoxycytidine, 5-Azacytidine, Cholecalciferol (Vitamin D3),  
Hydroxytamoxifen, Melatonin, Mifepristone, Raloxifene, all trans-Retinal  
(Vitamin A aldehyde), Retinoic acid, all trans (Vitamin A acid), 9-cis-  
15 Retinoic Acid, 13-cis-Retinoic acid, Retinol (Vitamin A), Tamoxifen,  
Troglitazone, and analogs and derivative thereof.

Examples of microtubule inhibitors include, but are not limited to,  
Colchicine, Dolastatin 15, Nocodazole, Paclitaxel, docetaxel,  
Podophyllotoxin, Rhizoxin, Vinblastine, Vincristine, Vinorelbine  
20 (Navelbine), and analogs and derivatives thereof.

Examples of other antitumor agents include, but are not limited to,  
17-(Allylamino)-17-demethoxygeldanamycin, 4-Amino-1,8-naphthalimide,  
Apigenin, Brefeldin A, Cimetidine, Dichloromethylene-diphosphonic acid,  
Leuprolide (Leuprorelin), Luteinizing Hormone-Releasing Hormone,  
25 Pifithrin- $\alpha$ , Rapamycin, Sex hormone-binding globulin, Thapsigargin,  
Urinary trypsin inhibitor fragment (Bikunin), and analogs and derivatives  
thereof.

## ii. Nucleic acids

In some embodiments, the agent is one or more nucleic acids. The  
30 nucleic acid can alter, correct, or replace an endogenous nucleic acid  
sequence. The nucleic acid is used to treat cancers, correct defects in genes  
in other pulmonary diseases and metabolic diseases affecting lung function,

genes such as those for the treatment of Parkinsons and ALS where the genes reach the brain through nasal delivery.

Gene therapy is a technique for correcting defective genes responsible for disease development. Researchers may use one of several approaches for correcting faulty genes: A normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene. An abnormal gene can be swapped for a normal gene through homologous recombination. The abnormal gene can be repaired through selective reverse mutation, which returns the gene to its normal function. The regulation (the degree to which a gene is turned on or off) of a particular gene can be altered.

The nucleic acid can be a DNA, RNA, a chemically modified nucleic acid, or combinations thereof. For example, methods for increasing stability of nucleic acid half-life and resistance to enzymatic cleavage are known in the art, and can include one or more modifications or substitutions to the nucleobases, sugars, or linkages of the polynucleotide. The nucleic acid can be custom synthesized to contain properties that are tailored to fit a desired use. Common modifications include, but are not limited to use of locked nucleic acids (LNAs), unlocked nucleic acids (UNAs), morpholinos, peptide nucleic acids (PNA), phosphorothioate linkages, phosphonoacetate linkages, propyne analogs, 2'-O-methyl RNA, 5-Me-dC, 2'-5' linked phosphodiester lineage, Chimeric Linkages (Mixed phosphorothioate and phosphodiester linkages and modifications), conjugation with lipid and peptides, and combinations thereof.

In some embodiments, the nucleic acid includes internucleotide linkage modifications such as phosphate analogs having achiral and uncharged intersubunit linkages (e.g., Sterchak, E. P. et al., *Organic Chem.*, 52:4202, (1987)), or uncharged morpholino-based polymers having achiral intersubunit linkages (see, e.g., U.S. Patent No. 5,034,506). Some internucleotide linkage analogs include morpholidate, acetal, and polyamide-linked heterocycles. Other backbone and linkage modifications include, but are not limited to, phosphorothioates, peptide nucleic acids, tricyclo-DNA,



decoy oligonucleotide, ribozymes, spiegelmers (containing L nucleic acids, an aptamer with high binding affinity), or CpG oligomers.

Phosphorothioates (or S-oligos) are a variant of normal DNA in which one of the nonbridging oxygens is replaced by a sulfur. The  
5 sulfurization of the internucleotide bond dramatically reduces the action of endo- and exonucleases including 5' to 3' and 3' to 5' DNA POL I exonuclease, nucleases S1 and P1, RNases, serum nucleases and snake venom phosphodiesterase. In addition, the potential for crossing the lipid bilayer increases. Because of these important improvements,  
10 phosphorothioates have found increasing application in cell regulation. Phosphorothioates are made by two principal routes: by the action of a solution of elemental sulfur in carbon disulfide on a hydrogen phosphonate, or by the more recent method of sulfurizing phosphite triesters with either tetraethylthiuram disulfide (TETD) or 3H-1, 2-benzodithiol-3-one 1, 1-  
15 dioxide (BDTD).<sup>4</sup> The latter methods avoid the problem of elemental sulfur's insolubility in most organic solvents and the toxicity of carbon disulfide. The TETD and BDTD methods also yield higher purity phosphorothioates.

Peptide nucleic acids (PNA) are molecules in which the phosphate  
20 backbone of oligonucleotides is replaced in its entirety by repeating N-(2-aminoethyl)-glycine units and phosphodiester bonds are replaced by peptide bonds. The various heterocyclic bases are linked to the backbone by methylene carbonyl bonds. PNAs maintain spacing of heterocyclic bases that is similar to oligonucleotides, but are achiral and neutrally charged  
25 molecules. Peptide nucleic acids are typically comprised of peptide nucleic acid monomers. The heterocyclic bases can be any of the standard bases (uracil, thymine, cytosine, adenine and guanine) or any of the modified heterocyclic bases described below. A PNA can also have one or more peptide or amino acid variations and modifications. Thus, the backbone  
30 constituents of PNAs may be peptide linkages, or alternatively, they may be non-peptide linkages. Examples include acetyl caps, amino spacers such as 8-amino-3,6-dioxaoctanoic acid (referred to herein as O-linkers), and the like. Methods for the chemical assembly of PNAs are well known.

In some embodiments, the nucleic acid includes one or more chemically-modified heterocyclic bases including, but are not limited to, inosine, 5-(1-propynyl) uracil (pU), 5-(1-propynyl) cytosine (pC), 5-methylcytosine, 8-oxo-adenine, pseudocytosine, pseudoisocytosine, 5 and 2-amino-5-(2'-deoxy- $\beta$ -D-ribofuranosyl)pyridine (2-aminopyridine), and various pyrrolo- and pyrazolopyrimidine derivatives, 4-acetylcytosine, 8-hydroxy-N-6-methyladenosine, aziridinylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methyl guanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxy-aminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, 2,6-diaminopurine, and 2'-modified analogs such as, but not limited to O-methyl, amino-, and fluoro-modified analogs. Inhibitory RNAs modified with 2'-fluoro (2'-F) pyrimidines appear to have favorable properties *in vitro*.

In some embodiments the nucleic acid includes one or more sugar moiety modifications, including, but are not limited to, 2'-O-aminoethoxy, 2'-O-aminoethyl (2'-OAE), 2'-O-methoxy, 2'-O-methyl, 2-guanidoethyl (2'-OGE), 2'-O,4'-C-methylene (LNA), 2'-O-(methoxyethyl) (2'-OME) and 2'-O-(N-(methyl)acetamido) (2'-OMA).

Methods of gene therapy typically rely on the introduction into the cell of a nucleic acid molecule that alters the genotype of the cell. Introduction of the nucleic acid molecule can correct, replace, or otherwise alters the endogenous gene via genetic recombination. Methods can include introduction of an entire replacement copy of a defective gene, a heterologous gene, or a small nucleic acid molecule such as an

oligonucleotide. This approach typically requires delivery systems to introduce the replacement gene into the cell, such as genetically engineered viral vectors.

Methods to construct expression vectors containing genetic sequences and appropriate transcriptional and translational control elements are well known in the art. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Expression vectors generally contain regulatory sequences necessary elements for the translation and/or transcription of the inserted coding sequence. For example, the coding sequence is preferably operably linked to a promoter and/or enhancer to help control the expression of the desired gene product. Promoters used in biotechnology are of different types according to the intended type of control of gene expression. They can be generally divided into constitutive promoters, tissue-specific or development-stage-specific promoters, inducible promoters, and synthetic promoters.

Viral vectors include adenovirus, adeno-associated virus, herpes virus, vaccinia virus, polio virus, AIDS virus, neuronal trophic virus, Sindbis and other RNA viruses, including these viruses with the HIV backbone. Also useful are any viral families which share the properties of these viruses which make them suitable for use as vectors. Typically, viral vectors contain, nonstructural early genes, structural late genes, an RNA polymerase III transcript, inverted terminal repeats necessary for replication and encapsidation, and promoters to control the transcription and replication of the viral genome. When engineered as vectors, viruses typically have one or more of the early genes removed and a gene or gene/promoter cassette is inserted into the viral genome in place of the removed viral DNA.

Gene targeting via target recombination, such as homologous recombination (HR), is another strategy for gene correction. Gene correction at a target locus can be mediated by donor DNA fragments homologous to the target gene (Hu, et al., Mol. Biotech., 29:197-210 (2005); Olsen, et al., J. Gene Med., 7:1534-1544 (2005)). One method of targeted recombination includes the use of triplex-forming oligonucleotides (TFOs) which bind as third strands to homopurine/homopyrimidine sites in duplex DNA in a

sequence-specific manner. Triplex forming oligonucleotides can interact with either double-stranded or single-stranded nucleic acids. When triplex molecules interact with a target region, a structure called a triplex is formed, in which there are three strands of DNA forming a complex dependent on both Watson-Crick and Hoogsteen base-pairing. Triplex molecules are preferred because they can bind target regions with high affinity and specificity. It is preferred that the triplex forming molecules bind the target molecule with a  $K_d$  less than  $10^{-6}$ ,  $10^{-8}$ ,  $10^{-10}$ , or  $10^{-12}$ . Methods for targeted gene therapy using triplex-forming oligonucleotides (TFO's) and peptide nucleic acids (PNAs) are described in U.S. Published Application No. 20070219122 and their use for treating infectious diseases such as HIV are described in U.S. Published Application No. 2008050920. The triplex-forming molecules can also be tail clamp peptide nucleic acids (tcPNAs), such as those described in U.S. Published Application No. 2011/0262406.

Double duplex-forming molecules, such as a pair of pseudocomplementary oligonucleotides, can also induce recombination with a donor oligonucleotide at a chromosomal site. Use of pseudocomplementary oligonucleotides in targeted gene therapy is described in U.S. Published Application No. 2011/0262406.

## **2. Diagnostic Agents**

Exemplary diagnostic materials include paramagnetic molecules, fluorescent compounds, magnetic molecules, and radionuclides. Suitable diagnostic agents include, but are not limited to, x-ray imaging agents and contrast media. Radionuclides also can be used as imaging agents. Examples of other suitable contrast agents include gases or gas emitting compounds, which are radioopaque. Liposomes can further include agents useful for determining the location of administered particles. Agents useful for this purpose include fluorescent tags, radionuclides and contrast agents.

## **3. CEST agents**

CEST agents are agents that can rapidly exchange protons with the surrounding water during an MRI. Examples of suitable CEST agents include, but are not limited to, L-arginine, barbituric acid, many analogs of barbituric acid with replacement of the hydrogen at the 5 position by an

organic group, glycogen, glucose, myoinositol, glutamate, creatine and many polycationic peptides (e.g., poly-L-lysine). In some embodiments, the agent provides an offset from water of at least 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, or greater from water. In particular embodiments, the offset is about 5.0 ppm.

- 5 In some embodiments, the agent is L-arginine, poly-L-lysine, or barbituric acid. In particular embodiments, the agent is barbituric acid.

### III. Pharmaceutical formulations

- For those embodiments where the one or more therapeutic, prophylactic, and/or diagnostic agents are encapsulated within a liposomal nanoparticle and/or associated with the surface of the liposome, the percent drug loading is from about 1% to about 80%, from about 1% to about 50%, preferably from about 1% to about 40% by weight, more preferably from about 1% to about 20% by weight, most preferably from about 1% to about 10% by weight. The ranges above are inclusive of all values from 1% to 80%. For those embodiments where the agent is associated with the surface of the particle, the percent loading may be higher since the amount of drug is not limited by the methods of encapsulation. In some embodiments, the agent to be delivered may be encapsulated within a liposome and associated with the surface of the particle.

- 20 The formulations described herein contain an effective amount of liposomes ("MPPs") in a pharmaceutical carrier appropriate for administration to a mucosal surface, wherein the pharmaceutical carrier is adjusted to be hypotonic. One skilled in the art can routinely adjust tonicity of pharmaceutical carriers, once the desired tissue to be treated is identified, based on the preferred tonicity ranges described herein.

- Tonicity is the 'effective osmolality' and is equal to the sum of the concentrations of the solutes which have the capacity to exert an osmotic force across the membrane. A number of different materials can be used to adjust tonicity. For example, the USP 29-NF 24 lists five excipients classified as "tonicity" agents, including dextrose, glycerin; potassium chloride; mannitol; and sodium chloride See, for example, United States Pharmacopeia Convention, Inc. *United States Pharmacopeia 29-National Formulary 24*. Rockville MD: U.S. Pharmacopeia Convention, Inc.; 2005:

3261; Day, A. Dextrose. In: Rowe RC, Sheskey PJ and Owen SC, eds. *Handbook of Pharmaceutical Excipients*. 5th ed. Washington DC: American Pharmaceutical Association; 2005: 231-233; Price JC. Glycerin. In: Rowe RC, Sheskey PJ and Owen SC, eds. *Handbook of Pharmaceutical Excipients*. 5th ed. Washington DC: American Pharmaceutical Association; 2005: 301-303; Price JC. Glycerin. In: Rowe RC, Sheskey PJ and Owen SC, eds. *Handbook of Pharmaceutical Excipients*. 5th ed. Washington DC: American Pharmaceutical Association; 2005: 301-303; Armstrong NA. Mannitol. In: Rowe RC, Sheskey PJ and Owen SC, eds. *Handbook of Pharmaceutical Excipients*. 5th ed. Washington DC: American Pharmaceutical Association; 2005: 449-453; Owen SC. Sodium Chloride. In: Rowe RC, Sheskey PJ and Owen SC, eds. *Handbook of Pharmaceutical Excipients*. 5th ed. Washington DC: American Pharmaceutical Association; 2005: 671-674. Mannitol is an example of a GRAS listed ingredient accepted for use as a food additive in Europe, included in the FDA Inactive Ingredients Database (IP, IM, IV, and SC injections; infusions; buccal, oral and sublingual tablets, powders and capsules; ophthalmic preparations; topical solutions), included in nonparenteral and parenteral medicines licensed in the UK and included in the Canadian Natural Health Products Ingredients Database. A 5.07% w/v aqueous solution is isoosmotic with serum.

Minimally hypotonic formulations, preferably ranging from 20-220 mOsm/kg, provide rapid and uniform delivery of MPP to the entire vaginal surface, with minimal risk of epithelial toxicity. There is a higher osmolality in the colon, such that vehicles with an osmolality above that of blood plasma (generally considered isotonic at ~300 mOsm/kg), leads to improvements in distribution in the colon. The range for improved colon distribution with a hypotonic vehicle in the colon is ~20 mOsm/kg-450 mOsm/kg.

#### **A. Pulmonary formulations**

Pharmaceutical formulations and methods for the pulmonary administration of active agents to patients are known in the art.

The respiratory tract is the structure involved in the exchange of gases between the atmosphere and the blood stream. The respiratory tract

encompasses the upper airways, including the oropharynx and larynx, followed by the lower airways, which include the trachea followed by bifurcations into the bronchi and bronchioli. The upper and lower airways are called the conducting airways. The terminal bronchioli then divide into  
5 respiratory bronchioli which then lead to the ultimate respiratory zone, the alveoli, or deep lung, where the exchange of gases occurs.

Formulations can be divided into dry powder formulations and liquid formulations. Both dry powder and liquid formulations can be used to form aerosol formulations. The term aerosol as used herein refers to any  
10 preparation of a fine mist of particles, which can be in solution or a suspension, whether or not it is produced using a propellant.

### **1. Dry Powder Formulations**

Dry powder formulations are finely divided solid formulations containing liposome carriers which are suitable for pulmonary  
15 administration. Dry powder formulations include, at a minimum, one or more liposome carriers which are suitable for pulmonary administration. Such dry powder formulations can be administered via pulmonary inhalation to a patient without the benefit of any carrier, other than air or a suitable propellant.

20 In other embodiments, the dry powder formulations contain one or more liposome gene carriers in combination with a pharmaceutically acceptable carrier. In these embodiments, the liposome gene carriers and pharmaceutical carrier can be formed into nano- or microparticles for delivery to the lung.

25 The pharmaceutical carrier may include a bulking agent or a lipid or surfactant. Natural surfactants such as dipalmitoylphosphatidylcholine (DPPC) are the most preferred. Synthetic and animal derived pulmonary surfactants include Exosurf - a mixture of DPPC with hexadecanol and tyloxapol added as spreading agents, Pumactant (Artificial Lung Expanding  
30 Compound or ALEC) - a mixture of DPPC and PG, KL-4 - composed of DPPC, palmitoyl-oleoyl phosphatidylglycerol, and palmitic acid, combined with a 21 amino acid synthetic peptide that mimics the structural characteristics of SP-B, Venticute - DPPC, PG, palmitic acid and

recombinant SP-C, Alveofact - extracted from cow lung lavage fluid, Curosurf - extracted from material derived from minced pig lung, Infasurf - extracted from calf lung lavage fluid, and Survanta - extracted from minced cow lung with additional DPPC, palmitic acid and tripalmitin. Exosurf, 5 Curosurf, Infasurf, and Survanta are the surfactants currently FDA approved for use in the U.S.

The pharmaceutical carrier may also include one or more stabilizing agents or dispersing agents. The pharmaceutical carrier may also include one or more pH adjusters or buffers. Suitable buffers include organic salts 10 prepared from organic acids and bases, such as sodium citrate or sodium ascorbate. The pharmaceutical carrier may also include one or more salts, such as sodium chloride or potassium chloride.

Dry powder formulations are typically prepared by blending one or more liposome carriers with one or more pharmaceutically acceptable 15 carriers. Optionally, additional active agents may be incorporated into the mixture as discussed below. The mixture is then formed into particles suitable for pulmonary administration using techniques known in the art, such as lyophilization, spray drying, agglomeration, spray coating, coacervation, low temperature casting, milling (*e.g.*, air-attrition milling (jet 20 milling), ball milling), high pressure homogenization, and/or supercritical fluid crystallization.

An appropriate method of particle formation can be selected based on the desired particle size, particle size distribution, and particle morphology desired for the formulation. In some cases, the method of particle formation 25 is selected so as to produce a population of particles with the desired particle size, particle size distribution for pulmonary administration. Alternatively, the method of particle formation can produce a population of particles from which a population of particles with the desired particle size, particle size distribution for pulmonary administration is isolated, for example by sieving.

30 It is known in the art that particle morphology affects the depth of penetration of a particle into the lung. Accordingly, dry powder formulations is processed into particles having the appropriate mass median aerodynamic diameter (MMAD), tap density, and surface roughness to



achieve delivery of the one or more active agents to the desired region(s) of the lung. For example, preferred particle morphologies for delivery to the deep lung are known in the art, and are described, for example, in U.S. Patent No. 7,052,678 to Vanbever, *et al.*

5           Particles having a mass median aerodynamic diameter (MMAD) of greater than about 5 microns generally do not reach the lung; instead, they tend to impact the back of the throat and are swallowed. Particles having diameters of about 3 to about 5 microns are small enough to reach the upper-  
10           to mid-pulmonary region (conducting airways), but may be too large to reach the alveoli. Smaller particles, (*i.e.*, about 0.5 to about 3 microns), are capable of efficiently reaching the alveolar region. Particles having diameters smaller than about 0.5 microns can also be deposited in the alveolar region by sedimentation.

            The precise particle size range effective to achieve delivery to the  
15           alveolar region will depend on several factors, including the tap density of particles being delivered. Generally speaking, as tap density decreases, the MMAD of particles capable of efficiently reaching the alveolar region of the lungs increases. Therefore, in cases of particles with low tap densities, particles having diameters of about 3 to about 5 microns, about 5 to about 7  
20           microns, or about 7 to about 9.5 microns can be efficiently delivered to the lungs. The preferred aerodynamic diameter for maximum deposition within the lungs can be calculated. See, for example, U.S. Patent No. 7,052,678 to Vanbever, *et al.*

            Microparticles cannot diffuse through mucus even if their surface is  
25           muco-resistant. However, mucus-penetrating particles can be encapsulated in microparticles to impact upper lung, and subsequently release the liposomes. In some cases, the particles are spherical or ovoid in shape. The particles can have a smooth or rough surface texture. The particles may also be coated with a polymer or other suitable material to control release of one or more  
30           active agents in the lungs.

            Dry powder formulations can be administered as dry powder using suitable methods known in the art. Alternatively, the dry powder formulations can be suspended in the liquid formulations described below,

and administered to the lung using methods known in the art for the delivery of liquid formulations.

## 2. Liquid Formulations

Liquid formulations contain one or more liposome carriers suspended  
5 in a liquid pharmaceutical carrier. Suitable liquid carriers include, but are not limited to water, saline, and other physiologically acceptable aqueous solutions containing salts and/or buffers, such as phosphate buffered saline (PBS), Ringer's solution, and isotonic sodium chloride, or any other aqueous solution acceptable for administration to an animal or human, which is  
10 adjusted to the desired hypotonicity.

Preferably, liquid formulations are isotonic relative to physiological fluids and of approximately the same pH, ranging e.g., from about pH 4.0 to about pH 7.4, more preferably from about pH 6.0 to pH 7.0. The liquid pharmaceutical carrier can include one or more physiologically compatible  
15 buffers, such as a phosphate buffers. One skilled in the art can readily determine a suitable saline content and pH for an aqueous solution for pulmonary administration.

Liquid formulations may include one or more suspending agents, such as cellulose derivatives, sodium alginate, polyvinylpyrrolidone, gum  
20 tragacanth, or lecithin. Liquid formulations may also include one or more preservatives, such as ethyl or *n*-propyl *p*-hydroxybenzoate.

In some cases the liquid formulation may contain one or more solvents that are low toxicity organic (i.e. nonaqueous) class 3 residual solvents, such as ethanol, acetone, ethyl acetate, tetrahydrofuran, ethyl ether,  
25 and propanol. These solvents can be selected based on their ability to readily aerosolize the formulation. Any such solvent included in the liquid formulation should not detrimentally react with the one or more active agents present in the liquid formulation. The solvent should be sufficiently volatile to enable formation of an aerosol of the solution or suspension. Additional  
30 solvents or aerosolizing agents, such as a freon, alcohol, glycol, polyglycol, or fatty acid, can also be included in the liquid formulation as desired to increase the volatility and/or alter the aerosolizing behavior of the solution or suspension.

Liquid formulations may also contain minor amounts of polymers, surfactants, or other excipients well known to those of the art. In this context, "minor amounts" means no excipients are present that might adversely affect uptake of the one or more active agents in the lungs.

5                                    **3.        Aerosol Formulations**

The dry powder and liquid formulations described above can be used to form aerosol formulations for pulmonary administration. Aerosols for the delivery of therapeutic agents to the respiratory tract are known in the art. The term aerosol as used herein refers to any preparation of a fine mist of  
10 solid or liquid particles suspended in a gas. In some cases, the gas may be a propellant; however, this is not required. Aerosols may be produced using a number of standard techniques, including as ultrasonication or high pressure treatment.

In some cases, a device is used to administer the formulations to the  
15 lungs. Suitable devices include, but are not limited to, dry powder inhalers, pressurized metered dose inhalers, nebulizers, and electrohydrodynamic aerosol devices. Inhalation can occur through the nose and/or the mouth of the patient. Administration can occur by self-administration of the formulation while inhaling or by administration of the formulation via a  
20 respirator to a patient on a respirator.

**B.        Topical and Ophthalmic Formulations**

Topical or enteral formulations can be prepared as aqueous compositions using techniques is known in the art. Typically, such compositions can be prepared as solutions or suspensions; solid forms  
25 suitable for using to prepare solutions or suspensions upon the addition of a reconstitution medium prior to injection; emulsions, such as water-in-oil (w/o) emulsions, oil-in-water (o/w) emulsions, and microemulsions thereof, liposomes, emulsomes, sprays, gels, creams or ointments.

The carrier can be a solvent or dispersion medium containing, for  
30 example, water, ethanol, one or more polyols (e.g., glycerol, propylene glycol, and liquid polyethylene glycol), oils, such as vegetable oils (e.g., peanut oil, corn oil, sesame oil, etc.), and combinations thereof. The proper fluidity can be maintained, for example, by the use of a coating, such as

lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants. In many cases, it will be preferable to include agents, for example, sugars or sodium chloride, to adjust the tonicity.

- 5           Solutions and dispersions of the active compounds as the free acid or base or pharmacologically acceptable salts thereof can be prepared in water or another solvent or dispersing medium suitably mixed with one or more pharmaceutically acceptable excipients including, but not limited to, surfactants, dispersants, emulsifiers, pH modifying agents, and combination  
10 thereof.

Suitable surfactants may be anionic, cationic, amphoteric or nonionic surface active agents. Suitable anionic surfactants include, but are not limited to, those containing carboxylate, sulfonate and sulfate ions. Examples of anionic surfactants include sodium, potassium, ammonium of long chain  
15 alkyl sulfonates and alkyl aryl sulfonates such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium bis-(2-ethylthioxyl)-sulfosuccinate; and alkyl sulfates such as sodium lauryl sulfate. Cationic surfactants include, but are not limited to, quaternary ammonium  
20 compounds such as benzalkonium chloride, benzethonium chloride, cetrimonium bromide, stearyl dimethylbenzyl ammonium chloride, polyoxyethylene and coconut amine. Examples of nonionic surfactants include ethylene glycol monostearate, propylene glycol myristate, glyceryl monostearate, glyceryl stearate, polyglyceryl-4-oleate, sorbitan acylate,  
25 sucrose acylate, PEG-150 laurate, PEG-400 monolaurate, polyoxyethylene monolaurate, polysorbates, polyoxyethylene octylphenylether, PEG-1000 cetyl ether, polyoxyethylene tridecyl ether, polypropylene glycol butyl ether, Poloxamer® 401, stearyl monoisopropanolamide, and polyoxyethylene hydrogenated tallow amide. Examples of amphoteric surfactants include  
30 sodium N-dodecyl- -alanine, sodium N-lauryl- -iminodipropionate, myristoamphoacetate, lauryl betaine and lauryl sulfobetaine.

The formulation can contain a preservative to prevent the growth of microorganisms. Suitable preservatives include, but are not limited to,

parabens, chlorobutanol, phenol, sorbic acid, and thimerosal. The formulation may also contain an antioxidant to prevent degradation of the active agent(s).

5 The formulation is typically buffered to a pH of 3-8 for administration upon reconstitution. Suitable buffers include, but are not limited to, phosphate buffers, acetate buffers, and citrate buffers.

Water soluble polymers are often used in pharmaceutical formulations. Suitable water-soluble polymers include, but are not limited to, polyvinylpyrrolidone, dextran, carboxymethylcellulose, and polyethylene  
10 glycol.

Sterile solutions can be prepared by incorporating the active compounds in the required amount in the appropriate solvent or dispersion medium with one or more of the excipients listed above, as required, followed by filtered sterilization. Generally, dispersions are prepared by  
15 incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those listed above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a  
20 powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The powders can be prepared in such a manner that the particles are porous in nature, which can increase dissolution of the particles. Methods for making porous particles are well known in the art.

25 Pharmaceutical formulations for ocular administration are preferably in the form of a sterile aqueous solution or suspension of particles formed from one or more polymer-drug conjugates. Acceptable solvents include, for example, water, Ringer's solution, phosphate buffered saline (PBS), and isotonic sodium chloride solution, which are then adjusted to the desired  
30 hypotonicity. The formulation may also be a sterile solution, suspension, or emulsion in a nontoxic, parenterally acceptable diluent or solvent such as 1,3-butanediol.

In some instances, the formulation is distributed or packaged in a liquid or semi-solid form such as a solution (eye drops), suspension, gel, cream or ointment. Alternatively, formulations for ocular administration can be packed as a solid, obtained, for example by lyophilization of a suitable liquid formulation. The solid can be reconstituted with an appropriate carrier or diluent prior to administration.

Solutions, suspensions, or emulsions for ocular administration may be buffered with an effective amount of buffer necessary to maintain a pH suitable for ocular administration. Suitable buffers are well known by those skilled in the art and some examples of useful buffers are acetate, borate, carbonate, citrate, and phosphate buffers. Solutions, suspensions, or emulsions for ocular administration may also contain one or more tonicity agents to adjust the isotonic range of the formulation. Suitable tonicity agents are well known in the art and some examples include glycerin, mannitol, sorbitol, sodium chloride, and other electrolytes.

Solutions, suspensions, or emulsions for ocular administration may also contain one or more preservatives to prevent bacterial contamination of the ophthalmic preparations. Suitable preservatives are known in the art, and include polyhexamethylenebiguanidine (PHMB), benzalkonium chloride (BAK), stabilized oxychloro complexes (otherwise known as Purite®), phenylmercuric acetate, chlorobutanol, sorbic acid, chlorhexidine, benzyl alcohol, parabens, thimerosal, and mixtures thereof.

Solutions, suspensions, or emulsions for ocular administration may also contain one or more excipients known in the art, such as dispersing agents, wetting agents, and suspending agents.

In still other embodiments, the liposomes are formulated for topical administration to mucosa. Suitable dosage forms for topical administration include creams, ointments, salves, sprays, gels, lotions, and emulsions. The compositions may contain one or more chemical penetration enhancers, membrane permeability agents, membrane transport agents, emollients, surfactants, stabilizers, and combination thereof.

In some embodiments, the liposomes can be administered as a liquid formulation, such as a solution or suspension, a semi-solid formulation, such

as a gel, a lotion or an ointment, or a solid formulation. A “gel” is a colloid in which the dispersed phase has combined with the continuous phase to produce a semisolid material, such as jelly.

In some embodiments, the liposomes are formulated as liquids,  
5 including solutions and suspensions, such as eye drops or as a semi-solid formulation, such as ointment or lotion for topical application to mucosa, such as the eye or vaginally or rectally.

The formulation may contain one or more excipients, such as emollients, surfactants, and emulsifiers.

10 “Emollients” are an externally applied agent that softens or soothes skin and are generally known in the art and listed in compendia, such as the “Handbook of Pharmaceutical Excipients”, 4<sup>th</sup> Ed., Pharmaceutical Press, 2003. These include, without limitation, almond oil, castor oil, ceratonia extract, cetostearyl alcohol, cetyl alcohol, cetyl esters wax, cholesterol,  
15 cottonseed oil, cyclomethicone, ethylene glycol palmitostearate, glycerin, glycerin monostearate, glyceryl monooleate, isopropyl myristate, isopropyl palmitate, lanolin, lecithin, light mineral oil, medium-chain triglycerides, mineral oil and lanolin alcohols, petrolatum, petrolatum and lanolin alcohols, soybean oil, starch, stearyl alcohol, sunflower oil, xylitol and combinations  
20 thereof. In one embodiment, the emollients are ethylhexylstearate and ethylhexyl palmitate.

“Surfactants” are surface-active agents that lower surface tension and thereby increase the emulsifying, foaming, dispersing, spreading and wetting properties of a product. Suitable non-ionic surfactants include emulsifying  
25 wax, glyceryl monooleate, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polysorbate, sorbitan esters, benzyl alcohol, benzyl benzoate, cyclodextrins, glycerin monostearate, poloxamer, povidone and combinations thereof. In one embodiment, the non-ionic surfactant is stearyl alcohol.

30 “Emulsifiers” are surface active substances which promote the suspension of one liquid in another and promote the formation of a stable mixture, or emulsion, of oil and water. Common emulsifiers are: metallic soaps, certain animal and vegetable oils, and various polar compounds.

Suitable emulsifiers include acacia, anionic emulsifying wax, calcium stearate, carbomers, cetostearyl alcohol, cetyl alcohol, cholesterol, diethanolamine, ethylene glycol palmitostearate, glycerin monostearate, glyceryl monooleate, hydroxypropyl cellulose, hypromellose, lanolin, hydrous, lanolin alcohols, lecithin, medium-chain triglycerides, methylcellulose, mineral oil and lanolin alcohols, monobasic sodium phosphate, monoethanolamine, nonionic emulsifying wax, oleic acid, poloxamer, poloxamers, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene stearates, propylene glycol alginate, self-emulsifying glyceryl monostearate, sodium citrate dehydrate, sodium lauryl sulfate, sorbitan esters, stearic acid, sunflower oil, tragacanth, triethanolamine, xanthan gum and combinations thereof. In one embodiment, the emulsifier is glycerol stearate.

Suitable classes of penetration enhancers are known in the art and include, but are not limited to, fatty alcohols, fatty acid esters, fatty acids, fatty alcohol ethers, amino acids, phospholipids, lecithins, cholate salts, enzymes, amines and amides, complexing agents (liposomes, cyclodextrins, modified celluloses, and diimides), macrocyclics, such as macrocyclic lactones, ketones, and anhydrides and cyclic ureas, surfactants, N-methyl pyrrolidones and derivatives thereof, DMSO and related compounds, ionic compounds, azone and related compounds, and solvents, such as alcohols, ketones, amides, polyols (e.g., glycols). Examples of these classes are known in the art.

An "oil" is a composition containing at least 95% wt of a lipophilic substance. Examples of lipophilic substances include but are not limited to naturally occurring and synthetic oils, fats, fatty acids, lecithins, triglycerides and combinations thereof.

A "continuous phase" refers to the liquid in which solids are suspended or droplets of another liquid are dispersed, and is sometimes called the external phase. This also refers to the fluid phase of a colloid within which solid or fluid particles are distributed. If the continuous phase is water (or another hydrophilic solvent), water-soluble or hydrophilic drugs



will dissolve in the continuous phase (as opposed to being dispersed). In a multiphase formulation (e.g., an emulsion), the discrete phase is suspended or dispersed in the continuous phase.

An "emulsion" is a composition containing a mixture of non-miscible components homogeneously blended together. In particular embodiments, the non-miscible components include a lipophilic component and an aqueous component. An emulsion is a preparation of one liquid distributed in small globules throughout the body of a second liquid. The dispersed liquid is the discontinuous phase, and the dispersion medium is the continuous phase.

When oil is the dispersed liquid and an aqueous solution is the continuous phase, it is known as an oil-in-water emulsion, whereas when water or aqueous solution is the dispersed phase and oil or oleaginous substance is the continuous phase, it is known as a water-in-oil emulsion. Either or both of the oil phase and the aqueous phase may contain one or more surfactants, emulsifiers, emulsion stabilizers, buffers, and other excipients. Preferred excipients include surfactants, especially non-ionic surfactants; emulsifying agents, especially emulsifying waxes; and liquid non-volatile non-aqueous materials, particularly glycols such as propylene glycol. The oil phase may contain other oily pharmaceutically approved excipients. For example, materials such as hydroxylated castor oil or sesame oil may be used in the oil phase as surfactants or emulsifiers.

An emulsion is a preparation of one liquid distributed in small globules throughout the body of a second liquid. The dispersed liquid is the discontinuous phase, and the dispersion medium is the continuous phase.

When oil is the dispersed liquid and an aqueous solution is the continuous phase, it is known as an oil-in-water emulsion, whereas when water or aqueous solution is the dispersed phase and oil or oleaginous substance is the continuous phase, it is known as a water-in-oil emulsion. The oil phase may consist at least in part of a propellant, such as an HFA propellant. Either or both of the oil phase and the aqueous phase may contain one or more surfactants, emulsifiers, emulsion stabilizers, buffers, and other excipients. Preferred excipients include surfactants, especially non-ionic surfactants; emulsifying agents, especially emulsifying waxes; and liquid non-volatile

non-aqueous materials, particularly glycols such as propylene glycol. The oil phase may contain other oily pharmaceutically approved excipients. For example, materials such as hydroxylated castor oil or sesame oil may be used in the oil phase as surfactants or emulsifiers.

5           A sub-set of emulsions are the self-emulsifying systems. These drug delivery systems are typically capsules (hard shell or soft shell) comprised of the drug dispersed or dissolved in a mixture of surfactant(s) and lipophilic liquids such as oils or other water immiscible liquids. When the capsule is exposed to an aqueous environment and the outer gelatin shell dissolves,  
10           contact between the aqueous medium and the capsule contents instantly generates very small emulsion droplets. These typically are in the size range of micelles or liposomes. No mixing force is required to generate the emulsion as is typically the case in emulsion formulation processes.

          A "lotion" is a low- to medium-viscosity liquid formulation. A lotion  
15           can contain finely powdered substances that are in soluble in the dispersion medium through the use of suspending agents and dispersing agents. Alternatively, lotions can have as the dispersed phase liquid substances that are immiscible with the vehicle and are usually dispersed by means of emulsifying agents or other suitable stabilizers. In one embodiment, the  
20           lotion is in the form of an emulsion having a viscosity of between 100 and 1000 centistokes. The fluidity of lotions permits rapid and uniform application over a wide surface area. Lotions are typically intended to dry on the skin leaving a thin coat of their medicinal components on the skin's surface.

25           A "cream" is a viscous liquid or semi-solid emulsion of either the "oil-in-water" or "water-in-oil type". Creams may contain emulsifying agents and/or other stabilizing agents. In one embodiment, the formulation is in the form of a cream having a viscosity of greater than 1000 centistokes, typically in the range of 20,000-50,000 centistokes. Creams are often time  
30           preferred over ointments as they are generally easier to spread and easier to remove.

          The difference between a cream and a lotion is the viscosity, which is dependent on the amount/use of various oils and the percentage of water

used to prepare the formulations. Creams are typically thicker than lotions, may have various uses and often one uses more varied oils/butters, depending upon the desired effect upon the skin. In a cream formulation, the water-base percentage is about 60-75 % and the oil-base is about 20-30 % of the total, with the other percentages being the emulsifier agent, preservatives and additives for a total of 100 %.

An "ointment" is a semisolid preparation containing an ointment base and optionally one or more active agents. Examples of suitable ointment bases include hydrocarbon bases (e.g., petrolatum, white petrolatum, yellow ointment, and mineral oil); absorption bases (hydrophilic petrolatum, anhydrous lanolin, lanolin, and cold cream); water-removable bases (e.g., hydrophilic ointment), and water-soluble bases (e.g., polyethylene glycol ointments). Pastes typically differ from ointments in that they contain a larger percentage of solids. Pastes are typically more absorptive and less greasy than ointments prepared with the same components.

A "gel" is a semisolid system containing dispersions of small or large molecules in a liquid vehicle that is rendered semisolid by the action of a thickening agent or polymeric material dissolved or suspended in the liquid vehicle. The liquid may include a lipophilic component, an aqueous component or both. Some emulsions may be gels or otherwise include a gel component. Some gels, however, are not emulsions because they do not contain a homogenized blend of immiscible components. Suitable gelling agents include, but are not limited to, modified celluloses, such as hydroxypropyl cellulose and hydroxyethyl cellulose; Carbopol homopolymers and copolymers; and combinations thereof. Suitable solvents in the liquid vehicle include, but are not limited to, diglycol monoethyl ether; alkylene glycols, such as propylene glycol; dimethyl isosorbide; alcohols, such as isopropyl alcohol and ethanol. The solvents are typically selected for their ability to dissolve the drug. Other additives, which improve the skin feel and/or emolliency of the formulation, may also be incorporated. Examples of such additives include, but are not limited, isopropyl myristate, ethyl acetate, C<sub>12</sub>-C<sub>15</sub> alkyl benzoates, mineral oil, squalane, cyclomethicone, capric/caprylic triglycerides, and combinations thereof.

Foams consist of an emulsion in combination with a gaseous propellant or gas-emitting component..

Buffers are used to control pH of a composition. Preferably, the buffers buffer the composition from a pH of about 4 to a pH of about 7.5, more preferably from a pH of about 4 to a pH of about 7, and most preferably from a pH of about 5 to a pH of about 7.

#### **D. Enteral Formulations**

Suitable oral dosage forms include tablets, capsules, solutions, suspensions, syrups, and lozenges. Tablets can be made using compression or molding techniques well known in the art. Gelatin or non-gelatin capsules can prepared as hard or soft capsule shells, which can encapsulate liquid, solid, and semi-solid fill materials, using techniques well known in the art. Formulations may be prepared using one or more pharmaceutically acceptable excipients, including diluents, preservatives, binders, lubricants, disintegrators, swelling agents, fillers, stabilizers, and combinations thereof.

Excipients, including plasticizers, pigments, colorants, stabilizing agents, and glidants, may also be used to form coated compositions for enteral administration. Delayed release dosage formulations may be prepared as described in standard references such as “Pharmaceutical dosage form tablets”, eds. Liberman et. al. (New York, Marcel Dekker, Inc., 1989), “Remington – The science and practice of pharmacy”, 20th ed., Lippincott Williams & Wilkins, Baltimore, MD, 2000, and “Pharmaceutical dosage forms and drug delivery systems”, 6th Edition, Ansel et al., (Media, PA: Williams and Wilkins, 1995). These references provide information on excipients, materials, equipment and process for preparing tablets and capsules and delayed release dosage forms of tablets, capsules, and granules.

The liposomes may be coated, for example to delay release once the particles have passed through the acidic environment of the stomach. Examples of suitable coating materials include, but are not limited to, cellulose polymers such as cellulose acetate phthalate, hydroxypropyl cellulose, hydroxypropyl methylcellulose, hydroxypropyl methylcellulose phthalate and hydroxypropyl methylcellulose acetate succinate; polyvinyl acetate phthalate, acrylic acid polymers and copolymers, and methacrylic

resins that are commercially available under the trade name EUDRAGIT® (Roth Pharma, Westerstadt, Germany), zein, shellac, and polysaccharides.

Diluents, also referred to as "fillers," are typically necessary to increase the bulk of a solid dosage form so that a practical size is provided for compression of tablets or formation of beads and granules. Suitable diluents include, but are not limited to, dicalcium phosphate dihydrate, calcium sulfate, lactose, sucrose, mannitol, sorbitol, cellulose, microcrystalline cellulose, kaolin, sodium chloride, dry starch, hydrolyzed starches, pregelatinized starch, silicone dioxide, titanium oxide, magnesium aluminum silicate and powdered sugar.

Binders are used to impart cohesive qualities to a solid dosage formulation, and thus ensure that a tablet or bead or granule remains intact after the formation of the dosage forms. Suitable binder materials include, but are not limited to, starch, pregelatinized starch, gelatin, sugars (including sucrose, glucose, dextrose, lactose and sorbitol), polyethylene glycol, waxes, natural and synthetic gums such as acacia, tragacanth, sodium alginate, cellulose, including hydroxypropylmethylcellulose, hydroxypropylcellulose, ethylcellulose, and veegum, and synthetic polymers such as acrylic acid and methacrylic acid copolymers, methacrylic acid copolymers, methyl methacrylate copolymers, aminoalkyl methacrylate copolymers, polyacrylic acid/polymethacrylic acid and polyvinylpyrrolidone.

Lubricants are used to facilitate tablet manufacture. Examples of suitable lubricants include, but are not limited to, magnesium stearate, calcium stearate, stearic acid, glycerol behenate, polyethylene glycol, talc, and mineral oil.

Disintegrants are used to facilitate dosage form disintegration or "breakup" after administration, and generally include, but are not limited to, starch, sodium starch glycolate, sodium carboxymethyl starch, sodium carboxymethylcellulose, hydroxypropyl cellulose, pregelatinized starch, clays, cellulose, alginine, gums or cross linked polymers, such as cross-linked PVP (Polyplasdone® XL from GAF Chemical Corp).

Stabilizers are used to inhibit or retard drug decomposition reactions that include, by way of example, oxidative reactions. Suitable stabilizers

include, but are not limited to, antioxidants, butylated hydroxytoluene (BHT); ascorbic acid, its salts and esters; Vitamin E, tocopherol and its salts; sulfites such as sodium metabisulphite; cysteine and its derivatives; citric acid; propyl gallate, and butylated hydroxyanisole (BHA).

5     **IV.     Methods of making mucus-penetrating liposomal particles**

10     The liposomes described herein can be prepared by a variety of techniques known in the art. The method selected is dependent on a variety of factors, such as: (1) the physicochemical characteristics of the material to be entrapped and those of the liposomal ingredients; (2) the nature of the medium in which the lipid vesicles are dispersed; (3) the effective concentration of the entrapped substance and its potential toxicity; (4) additional processes involved during application/delivery of the vesicles; (5) optimum size, polydispersity and shelf-life of the vesicles for the intended application; and (6) batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.

15     Formation of liposomes and nanoliposomes is not a spontaneous process. Lipid vesicles are formed when phospholipids such as lecithin are placed in water and consequently form one bilayer or a series of bilayers, each separated by water molecules, once enough energy is supplied.

20     Liposomes can be created by sonicating phosphatidylcholine rich phospholipids in water. Low shear rates create multilamellar liposomes, which have many layers like an onion.

25     Continued high-shear sonication tends to form smaller unilamellar liposomes. In this technique, the liposome contents are the same as the contents of the aqueous phase. Sonication is generally considered a "gross" method of preparation as it can damage the structure of the drug to be encapsulated. Newer methods such as extrusion and Mozafari method are employed to produce materials for animal (e.g., human) use.

30     In some embodiments, liposomal particles are prepared using a conventional thin film hydration and extrusion method. The lipid, PEG-conjugated lipid, and stabilizer, if present, are dissolved in an organic solvent (e.g., chloroform) at pre-determined molar ratios. A small proportion of a labeled-lipid, such as rhodamine labeled PE (Rho-PE), can be added to the

mixture to enable visualization of the liposomal particles via fluorescence microscopy. The mixture is placed in a rotavap with reduced atmosphere pressure to evaporate the organic solvent. The resulting lipid film is hydrated, such as with phosphate buffered saline (PBS), while agitated using  
5 a water bath sonicator to form multi-lamellar vehicles (MLV). The suspension is subsequently extruded through polycarbonate filters with pore sizes of 400 nm and 200 nm to generate unilamellar vehicles (LUV, i.e., liposomes with single bilayer membrane).

#### **IV. Methods of using mucus-penetrating liposomal particles**

10 The mucus-penetrating liposomal nanoparticles described herein can be used to deliver a variety of therapeutic, prophylactic, and/or diagnostic agents. In some embodiments, the agent or agents are hydrophilic or water-soluble, such as hydrophilic small molecule drugs, monoclonal antibodies and other protein-derived products. These agents are difficult to load into  
15 polymeric nanoparticles due to the hydrophobic nature of the particle core. This hydrophobic core is necessary in order to facilitate particle formation. In contrast, the lipid-based MPP described herein can be readily loaded with hydrophilic and hydrophobic substances. When particles are formulated, lipids self-assemble into bilayer membranes, forming a lipophilic shell for  
20 hydrophobic payloads, and entrapping an aqueous solution with hydrophilic payloads in the center.

The drug-loaded lipid-based particles described herein are able to diffuse rapidly and remain integral in human cervicovaginal mucus (CVM). The lipid-based MPP are expected to cross the mucus barrier timely to avoid  
25 aggregation or removal by mucus, and thus can distribute more uniformly, stay longer and provide more effective treatment for diseases on mucosal tissues, such as cervical cancers and sexually transmitted diseases (STD) as well as diseases of the colon, nose, lungs, and eyes.

The particles described here can also be used to provide an improved  
30 method for evaluating temporal retention and spatial distribution of the particles in the animal. Previous methods to evaluate the temporal retention and spatial distribution of nanocarriers dosed in animals mostly required labeling the carriers fluorescently and dissecting the animals for observation

and quantification. In contrast, by encapsulating a CEST MRI contrast agent, in the lipid-based MPP, the carriers were monitored using CEST MRI tools in live animals over time.

**A. Chemical Exchange Saturation Transfer (CEST)**

5 Chemical exchange saturation transfer (CEST) is a magnetic resonance imaging (MRI) contrast enhancement technique that enables indirect detection of contrast agents with exchangeable protons. Contrast agents with exchangeable protons including, but not limited to, L-arginine, barbituric acid, many analogs of barbituric acid with replacement of the  
10 hydrogen at the 5 position by an organic group, glycogen, glucose, myoinositol, glutamate, creatine and many polycationic peptides have been identified as potential *in vivo* CEST agents. Exogenous agents, such as diagnostic agents or contrast agents, can also be used for *in vivo* imaging. CEST technology has a number of indispensable features, such as the  
15 possibility of simultaneous detection of multiple ‘colors’ of agents and of changes in their environment (e.g. pH, metabolites, etc.) through MR contrast.

CEST exploits the ability of Nuclear Magnetic Resonance (NMR) to resolve different signals arising from protons on different molecules. By  
20 selectively saturating a particular proton signal (associated with a particular molecule or CEST agent) that is in exchange with surrounding water molecules, the MRI signal from the surrounding bulk water molecules is also attenuated. Images obtained with and without the RF saturating pulse reveal the location of the CEST agent. The chemical exchange must be in the  
25 intermediate regime where exchange is fast enough to efficiently saturate the bulk water signal but slow enough that there is a chemical shift difference between the exchangeable proton and the water proton resonances. The magnitude of the CEST effect therefore depends on both the exchange rate and the number of exchangeable protons.

30 CEST has three main advantages over traditional molecular imaging techniques: (1) the image contrast is controlled with radio-frequency (RF) pulses and can be turned on/off at will; (2) The molecules of interest, in some cases, can be directly detected, eliminating the need for contrast agent



to be delivered to, and to specifically react with, the molecule of interest; (3)  
A variant of the CEST technique, known as PARACEST, may be much more  
sensitive than traditional molecular imaging techniques and should be able to  
detect nanomolar concentrations. PARACEST typically relies on water  
5 exchange between the bulk water and water bound to paramagnetic  
Lanthanide complexes. Saturation of the Lanthanide ion bound water  
resonance leads to attenuation of the bulk water signal via water exchange.  
The large paramagnetic chemical shift of the bound water molecules allows  
them to tolerate much faster exchange rates with the bulk water while still  
10 while still remaining in the intermediate exchange regime, thereby providing  
much more efficient saturation of the bulk water signal and much greater  
CEST sensitivity.

CEST can be used to monitor drug delivery at a desired site from the  
mucus-penetrating liposomal particles described herein. For example, CEST  
15 can be used to monitor the vaginal drug delivery of liposomes for local  
treatment of cervical tumors by coencapsulating diamagnetic CEST contrast  
agents and drugs within the liposomal particles described herein. *In vitro*  
and *in vivo* images with PEG-coated liposomes show frequency offsets  
further from water (at 5 ppm) than previous designs. The integration of  
20 CEST and pegylated liposomal nanoparticles provides a non-invasive and  
quantitative way to evaluate the performance of these particles in vivo in  
preclinical studies.

### Examples

#### Example 1. Preparation of mucus-penetrating liposomal particles

25 Liposomal particles were prepared using a conventional thin film  
hydration and extrusion method. 12 mg of a mixture of phosphatidylcholine  
(PC, e.g., egg PC or hydrogenated soy PC), PEG-phosphatidylethanolamine  
(PEG-PE) and cholesterol (CHOL) were dissolved in chloroform at pre-  
determined molar ratios. A small proportion of Rhodamine labeled PE (Rho-  
30 PE) was added to the mixture to enable visualization of the liposomal  
particles via fluorescence microscopy. The mixture was placed in a rotavap  
with reduced atmosphere pressure to evaporate the organic solvent. The  
resulting lipid film was hydrated with phosphate buffered saline (PBS) while

agitated using a water bath sonicator to form multi-lamellar vehicles (MLV). The suspension was subsequently extruded through polycarbonate filters with pore sizes of 400 nm and 200 nm to generate unilamellar vehicles (LUV, i.e., liposomes with single bilayer membrane).

5 To load CEST agent (barbituric acid (BA)), the lipid thin film was hydrated with PBS buffer containing 25 mg/mL BA before annealing and agitation via sonication. To load drug (doxorubicin (DOX)), the MLV suspension was mixed with PBS buffer containing 2 mg/mL DOX before sonication and extrusion.

10 Though the thin film hydration and extrusion method was employed, liposomal particles can be prepared using other methods known in the art and should exhibit similar properties as long as the composition of the liposomal particles does not change.

A scheme of the composition of liposomal particles is shown in  
15 Figure. 1.

#### **Example 2. Characterization of liposomal particles**

The size and zeta-potential of liposomal particles were measured using dynamic light scattering and laser Doppler electrophoresis, respectively. Data are shown in Table 2.

20 **Table 2.** Compositions of liposomal particles and their basic characterization

<b>Molar Ratio (%)</b> (PC:CHOL:Rho-PE:PEG-PE)	<b>Sample</b> <b>Abbreviation</b>	<b>Size</b> <b>(Z-ave)</b> <b>(d. nm)</b>	<b>Polydispersity</b> <b>Index</b>	<b>Zeta Potential</b> <b>(mV)</b>
0.28:0.72:0.002:0.00	0% PEG	222	0.41	-2.5
0.26:0.68:0.002:0.05	5% PEG	160	0.16	-2.0
0.25:0.65:0.002:0.10	10% PEG	175	0.06	-2.2
0.22:0.58:0.002:0.20	20% PEG	132	0.15	-1.6

The size of the liposomal particles of all tested compositions ranged from 130 to 230 nm, well below the mesh dimension of human CVM  
25 (estimated to be >500nm). Higher ratio of PEG-conjugated lipids reduced the size of the particles, likely due to the formation of liposomal micelles. A

high proportion of cholesterol was used to enhance the stability of the liposomal particles.

**Example 3. Stability of mucus-penetrating liposomal particles**

To test the stability of the liposomal particles of different compositions, we incubated the particles in phosphate buffered saline (PBS) at 37°C and monitored the change of size and polydispersity over time, as shown in Figure 2. The size and polydispersity index (PDI) of the particles were steady for all the experimental groups for over 48 hrs. However, the PDI of liposomal particles with no PEG was consistently higher than other experimental group, indicating their poorer quality and weaker stability as compared to the PEGylated ones.

**Example 4. Diffusivity and stability of liposomal particles in mucus**

The diffusivity of the liposomal particles with different compositions in human CVM samples was evaluated. 0.6µl of diluted liposomal particles were added to 20µl freshly collected, undiluted human CVM. Samples were stored at room temperature and at different time points, 20 sec movies were acquired to record the trajectory of the particles. The qualitative observation of the mobility and the stability of the liposomal particles are summarized in Table 3.

**Table 3.** Compositions of liposomal particles and their basic characterization

Sample	Proportion of moving particles (Qualitative Observation)		Stability
	2hr*	15hr*	
0% PEG	0%	0%	Heavy aggregation
5% PEG	20%	~0-10%	Aggregation
10% PEG	80%	~30-70%	Some aggregation
20% PEG	~100%	~90-100%	Well dispersed

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\* Time post sample preparation.

Liposomal particles without any PEG coating were strongly immobilized and heavily aggregated by mucus. Incorporating PEG-PE facilitated the diffusion as well as the stability of the particles in mucus. The higher the proportion of PEG-PE, the more liposomal particles were freely diffusive, and more uniformly dispersed in mucus. Specifically, liposomal particles with 10% or 20% PEG remained mostly mobile and intact in mucus for at least 15 hr, long enough for particles to penetrate the mucus layers and reach the underlying epithelium. "Mobile" was defined as those particles which traveled a distance of over 10 times its radius (about 100 nm) in a 20 second movie, which corresponds to  $0.1 \mu\text{m}^2/\text{sec}$ .

Typically, PEGylated liposomal particles (e.g., stealth liposomes for intravenous injection) are composed of ~ 3 to 7 mol% PEG-conjugated lipid molecules. However, to enable particles to penetrate human mucus, it was found that a higher proportion of PEG (i.e., 10-20 mol%) is necessary.

**Example 5. Evaluation of mucus-penetrating liposomal particles for Chemical Exchange Saturation Transfer (CEST)**

*Animal Preparation*

C57BL/6 mice were given Depo-Provera (3 mg/mouse) by subcutaneous injection on the right flank 4 days prior to tumor inoculation. Luciferase expressing C3.34 cells were then implanted into the intravaginal cavity of mice, and bioluminescence imaging was used to monitor the growth of the tumor.

*CEST imaging*

Mice were anesthetized using isoflurane and positioned in a 11.7T horizontal bore Bruker Biospec scanner, and were imaged before and after intravaginal administration of 20  $\mu\text{l}$  of BA/ DOX PEGylated liposomes.

CEST images were acquired through collection of two sets of saturation images, a water saturation shift referencing (WASSR) set for B0 mapping and a CEST data set for characterizing contrast. For the CEST images:  $t_{\text{sat}}=3$  sec,  $B_1=4.7$  uT,  $TR=5$  sec, with offset incremented from -6 to +6 ppm (0.3 ppm steps) with a fat suppression pulse. The acquisition parameters were:  $TR=5.0$  sec, effective  $TE=21.6$  ms, RARE factor=8. The

CEST images were acquired every 30 min after the liposome administration up to 2 hrs.

#### *Data Analysis*

MR images were processed using custom-written Matlab scripts with  
5 the CEST contrast was the magnetization transfer ratio (for NH  
pMTRasym% =  $(S - \Delta\omega - S\Delta\omega)/S_0 \times 100\%$ ) protons at  $\Delta\omega = 5\text{ppm}$ .

Figure 3 is a representation of the rationale for designing liposomal nanoparticles for imaging particle-based therapy of cervical tumors.

The results of the *in vitro* CEST imaging for various liposomal  
10 particles are shown in Figure 4. The *in vitro* CEST contrast for these  
liposomes coated with 0%, 5%, 10% and 20% PEG was 53%, 27%, 25% and  
23% respectively at 5 ppm. These particles were stable over 2 days *in vitro*  
with no significant change in the size and PDI as described above.

Figure 4A shows the optimization of CEST contrast for liposomal  
15 particles containing 30, 50, and 70 mol percent cholesterol. Liposomes with  
>10% PEG and 50 mol% of cholesterol were stable and have high CEST  
contrast. The CEST liposomes could be visualized using MRI after local  
administration and their distribution could be traced over 2 hrs. The  
liposomes were located close to the opening of the vaginal cavity (ROI1),  
20 and seemed to stay in a distal region (ROI2) inside the vagina. The contrast  
in ROI1 and ROI2 increased right after administration and over the first 60  
min. Interestingly, the contrast in ROI2 was much higher than that in ROI1  
at 90 min post-administration, and it is higher than that of pre-administration.  
This indicated that liposomes could be retained inside the vagina for at least  
25 2 hrs.

We claim:

1. Mucus penetrating liposomal nanoparticles comprising one or more PEG-conjugated lipids, one or more lipids, and one or more therapeutic, prophylactic, and/or diagnostic agents, wherein at least 50% of the particles are mobile in freshly collected, undiluted human CVM at 2 hours and at least 30% of the particles are mobile in freshly collected, undiluted human CVM at 15 hours.
2. The particles of claim 1, wherein the concentration of the one or more PEG-conjugated lipids is at least about 8 mole percent.
3. The particles of claim 2, wherein the concentration of the one or more PEG-conjugated lipids is from about 8 mole percent to about 30 mole percent.
4. The particles of claim 3, wherein the concentration of the one or more PEG-conjugated lipids is from about 10 mole percent to about 20 mole percent.
5. The particles of any one of claims 1-4, wherein the one or more PEG-conjugated lipids is a PEG-conjugated phospholipid.
6. The particles of claim 5, wherein the PEG-conjugated phospholipid is selected from the group consisting of one or more of the lipids in Table 1.
7. The particles of claim 6, wherein the PEG-conjugated phospholipid is PEG-phosphatidylethanolamine (PEG-PE).
8. The particles of any one of claims 1-7, wherein the one or more lipids are selected from the group consisting of fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides (derived from condensation of ketoacyl subunits); and sterol lipids and prenol lipids (derived from condensation of isoprene subunits).
9. The particles of claim 8, wherein the one or more lipids is a phospholipid.
10. The particles of claim 9, wherein the phospholipid is phosphatidylcholine (PC).
11. The particles of any one of claims 1-10, wherein the particles further comprise one or more stabilizers.
12. The particles of claim 11, wherein the stabilizer is cholesterol.

13. The particles of any one of claims 1-12, wherein the concentration of the stabilizer is from about 10 mole percent to about 40 mole percent, preferably about 20 mole percent to about 40 mole percent, more preferably from about 30 mole percent to about 40 mole percent.
14. The particles of any one of claims 1-13, wherein the particles further comprise a therapeutic agent, prophylactic agent, diagnostic agent, or combinations thereof.
15. The particles of claim 14, wherein the agent is a therapeutic agent.
16. The particles of claim 15, wherein the therapeutic agent is an antitumor agent.
17. The particles of claim 16, wherein the antitumor agent is doxorubicin.
18. The particles of any one of claims 1-17, further comprising a CEST agent.
19. The particles of claim 18, wherein the CEST agent is barbituric acid.
20. The particles of any one of claims 1-19, wherein the particle further comprises an imaging agent.
21. A method for administering a therapeutic, prophylactic, and/or diagnostic agent to a patient in need thereof, the method comprising administering an effective amount of the mucus-penetrating liposomal nanoparticles of any one of claims 1-20.

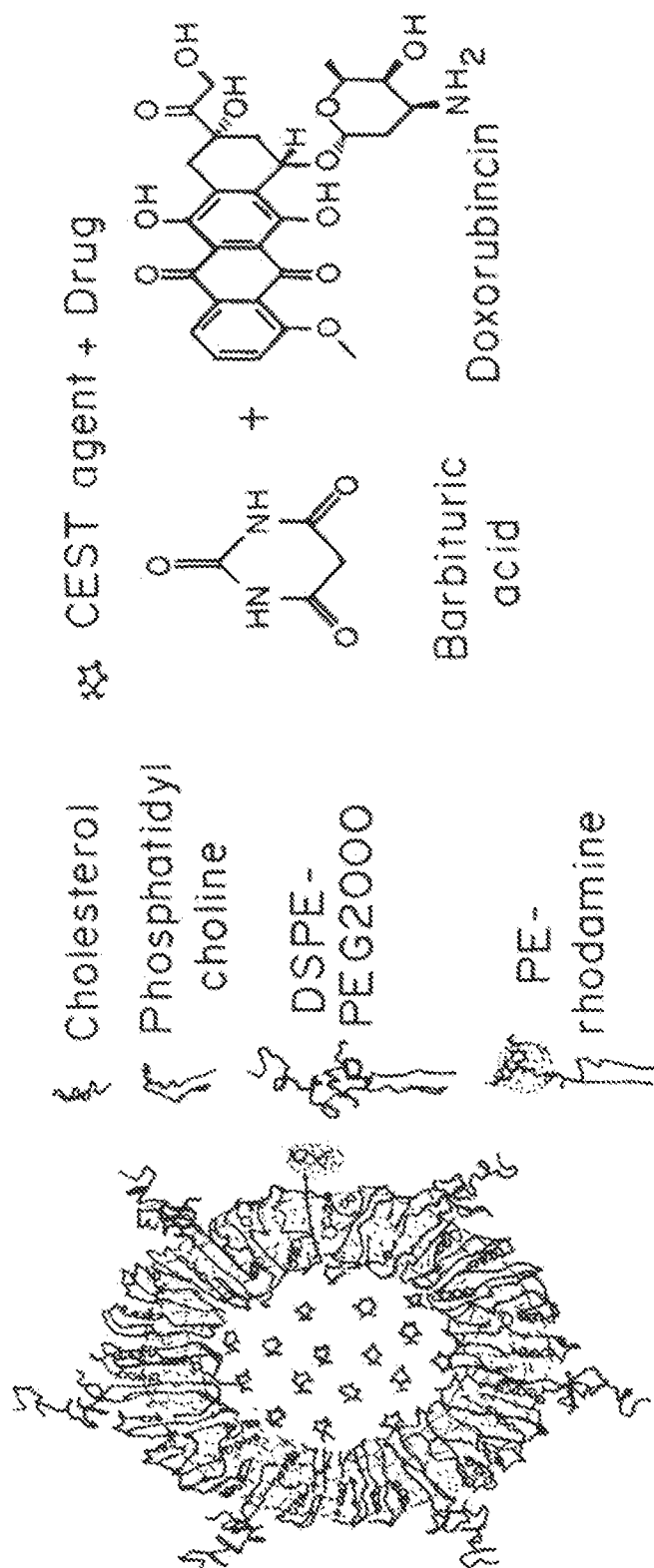
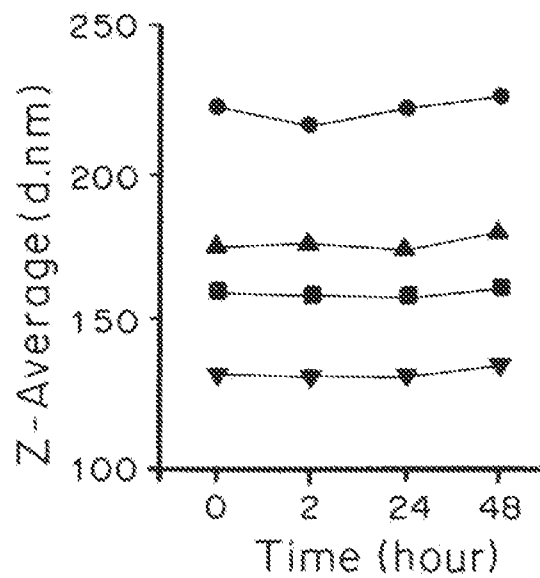
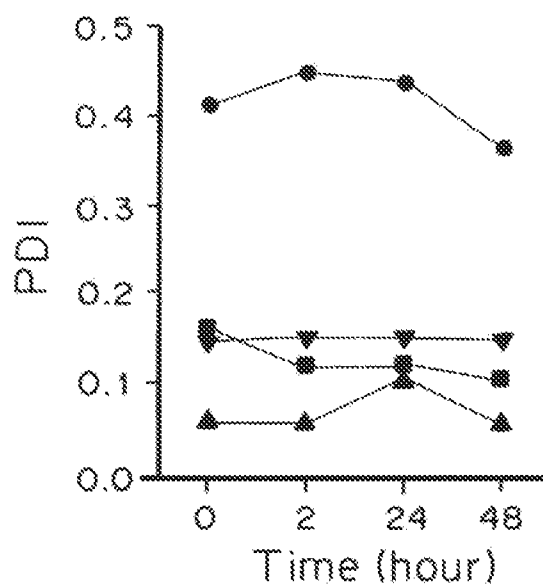
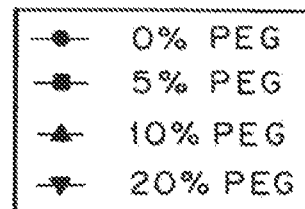


FIG. 7



*FIG. 2A**FIG. 2B*

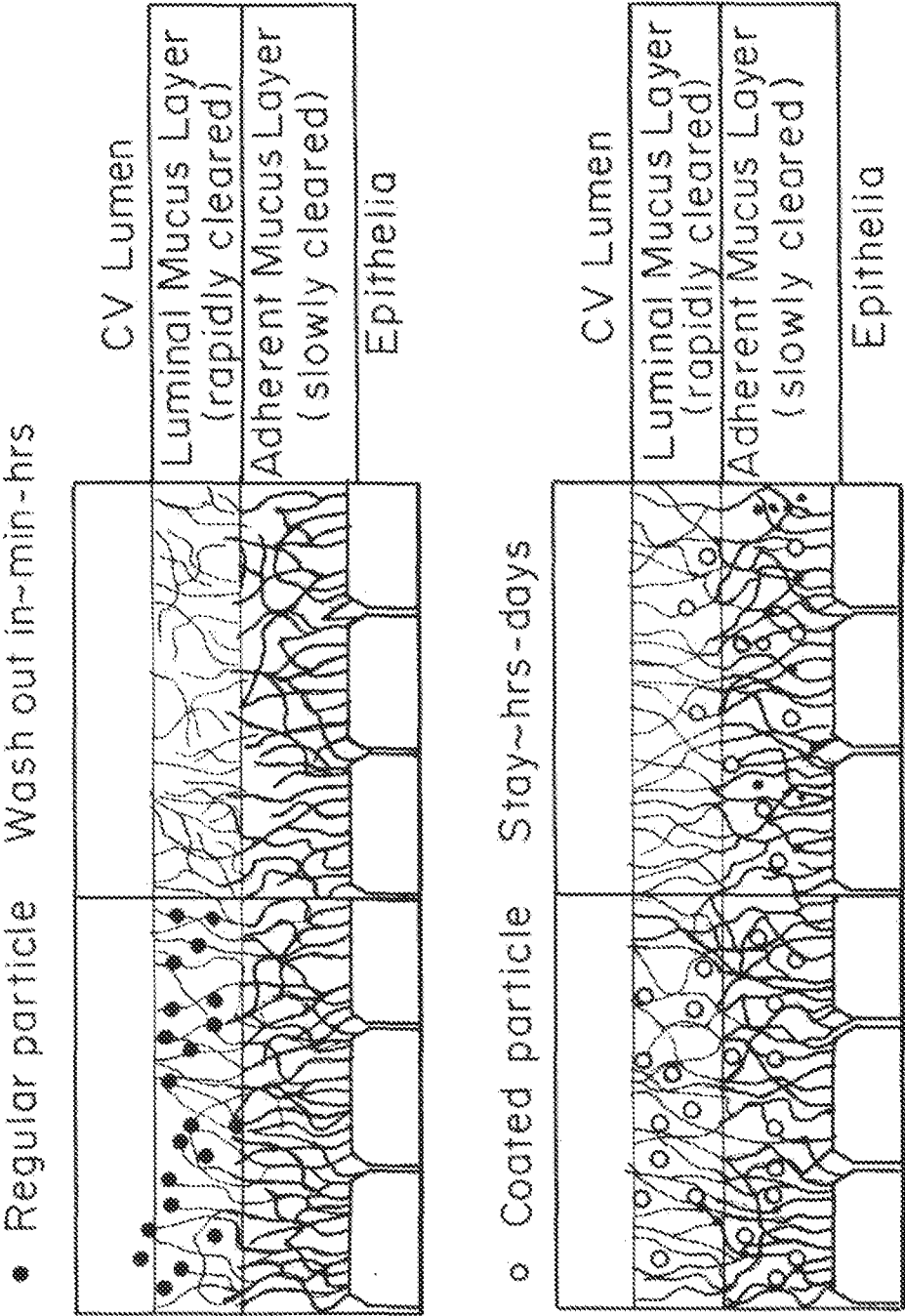
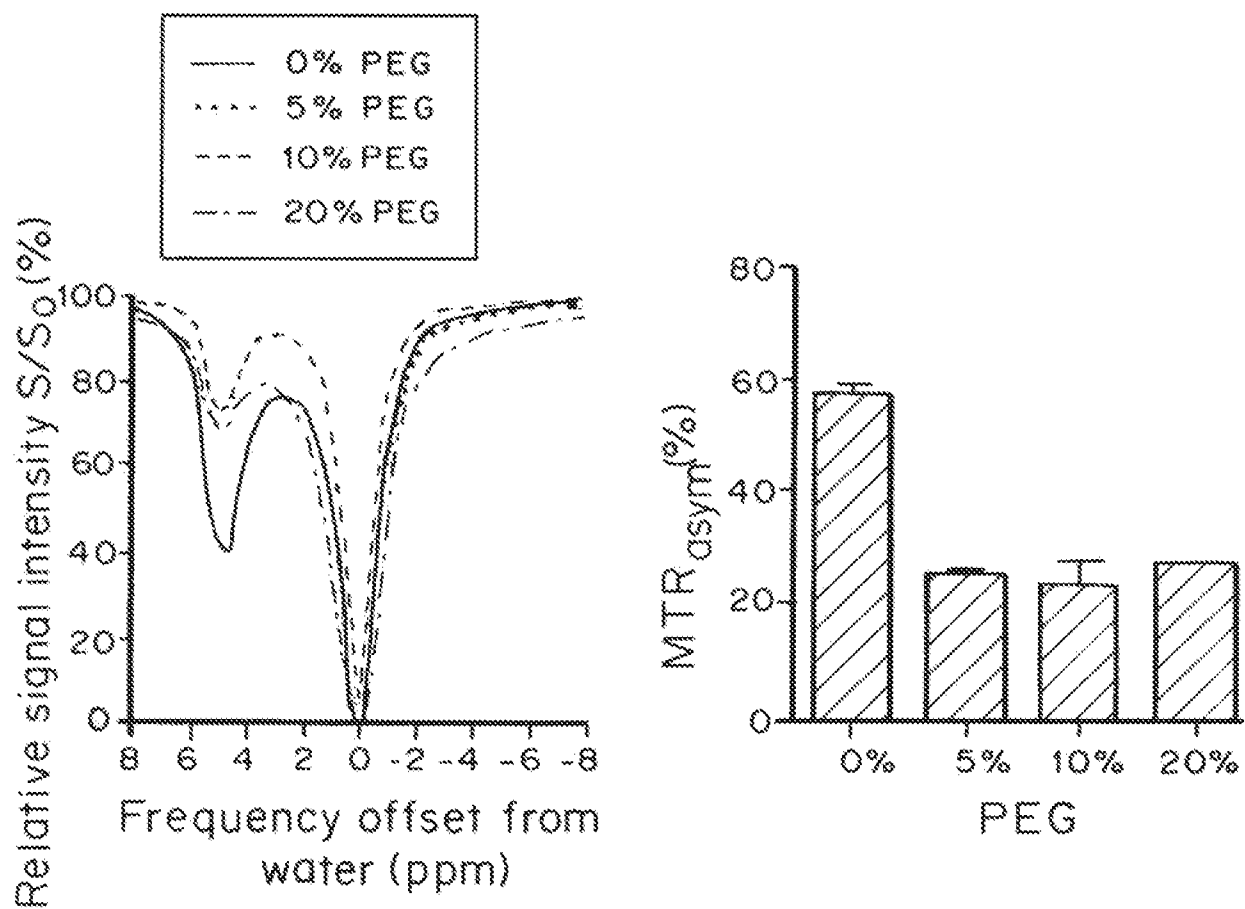


FIG. 3

**FIG. 4**

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/US2013/039731

A. CLASSIFICATION OF SUBJECT MATTER  
INV. A61K9/127  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE, MEDLINE, COMPENDEX, INSPEC

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CATTEL L ET AL: "FROM CONVENTIONAL TO STEALTH LIPOSOMES A NEW FRONTIER IN CANCER CHEMOTHERAPY", TUMORI, IT, vol. 89, no. 3, 1 January 2003 (2003-01-01), pages 237-249, XP008069891, ISSN: 0300-8916 figure 8 abstract	1-21
X	US 2010/063135 A1 (DANDE PRASAD A [US] ET AL) 11 March 2010 (2010-03-11) abstract paragraphs [0005] - [0007] paragraph [0052] pages -; claims ----- -/--	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

31 July 2013

Date of mailing of the international search report

02/09/2013

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## INTERNATIONAL SEARCH REPORT

International application No

PCT/US2013/039731

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 2008/030557 A2 (UNIV JOHNS HOPKINS [US]; HANES JUSTIN [US]; LAI SAMUEL K [US]) 13 March 2008 (2008-03-13) the whole document -----	1-21

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Information on patent family members

International application No

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